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**STUDIES OF THE GENES AND ENZYMES OF
THE SHIKIMATE PATHWAY IN**

Streptomyces coelicolor.

Graeme Edward Walker

Thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy

Department of Biochemistry

September, 1991

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The abbreviations used are those recommended in the Biochemical Journal "Instructions to Authors", except the following :-

| | |
|-------------------|--|
| Amp. | ampicillin |
| ATP | adenosine triphosphate |
| Ax | absorbance at x nm measured with a 1cm path |
| Bis-trispropane | 1,3-bis (tris(Hydroxymethyl) methylamino)-propane |
| BSA | bovine serum albumin |
| (k)Da | (kilo) daltons |
| DAHP | 3-deoxy-D- <i>arabino</i> -heptulosonate 7-phosphate |
| DEAE | diethyl aminoethyl |
| DHQ | 3-dehydroquinate |
| DMSO | dimethylsulphoxide |
| DNAse | deoxyribonuclease |
| DTNB | 5,5'-dithio-bis (2-nitrobenzoic acid) |
| DTT | dithiothreitol |
| E4P | erythrose 4-phosphate |
| EDTA | ethylene diamine tetra acetate |
| EPSP | 5- <i>enol</i> pyruvyl-shikimate 3-phosphate |
| EtBr | ethidium bromide |
| f.p.l.c. | fast protein liquid chromatography |
| h.p.l.c. | high pressure liquid chromatography |
| IPTG | isopropyl- β -D-thiogalactoside |
| kb | kilo base pairs |
| MES | 2-(N-Morpholino) ethanesulphonic acid |
| Mr | molecular weight |
| NAD ⁺ | nicotinamide adenine dinucleotide |
| NADP ⁺ | nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| ODx | optical density at x nm measured with a 1cm path |
| PAGE | polyacrylamide gel electrophoresis |
| PEP | phospho <i>enol</i> pyruvate |
| pfu | plaque forming units |
| PITC | phenyl isothiocyanate |
| PMSF | phenylmethanesulphonylfluoride |
| PTH | phenylthiohydantoin |

| | |
|-------|---|
| RNAse | ribonuclease |
| SDS | sodium dodecyl sulphate |
| SSC | standard saline citrate |
| TAE | Tris-acetate/EDTA buffer |
| TBE | Tris-borate/EDTA buffer |
| TEMED | N,N,N',N'-tetramethylethylene diamine |
| TES | N-tris (Hydroxymethyl) methyl-2-aminoethane sulphonic acid |
| Tris | Tris (hydroxymethyl) aminomethane |
| (m)U | (milli)units of enzyme activity |
| UV | ultra violet |
| Ve | elution volume |
| X-gal | 5-bromo-4-chloro-3-indoyl- β -galactoside |

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SUMMARY

Aromatic compounds are synthesized via the shikimate pathway. The first activity, DAHP synthase, has been implicated in the regulation of the pathway in a number of organisms. This activity was investigated in the organism *S.coelicolor* and an assessment made of its regulatory importance.

Methods have been developed for the purification of the *S.coelicolor* DAHP synthase to obtain electrophoretically homogeneous enzyme. The purified *S.coelicolor* DAHP synthase has a native M_r of 107 000 with a subunit M_r of 54 000 and appears likely to be a homodimer.

In minimal medium-grown cells a single DAHP synthase activity was detected, making it unlikely that *S.coelicolor* possesses isoenzyme forms. This activity was subject to feedback inhibition by the pathway end-product tryptophan, but was unaffected by phenylalanine or tyrosine, or the pathway intermediates *p*-aminobenzoate and anthranilate. Preliminary evidence suggests that, in common with DAHP synthases from other organisms, a thiol group or a metal ion are essential for activity. Repression control appeared to be absent as this activity was constitutively expressed in complex medium-grown cells.

The *N*-terminal sequence of the *S.coelicolor* DAHP synthase has been determined for the first twenty-six residues. The sequence of internal peptides has also been determined. The *S.coelicolor* DAHP synthase *N*-terminal sequence does not resemble that from *E.coli*, *S.cerevisiae* or potato.

These sequences were used to design oligonucleotide probes corresponding to regions of the *S.coelicolor* DAHP synthase structural gene. These probes were then used to screen a genomic sub-library constructed in pUC18 and a genomic library constructed in λ GEM-11. Two positive signals were isolated from the λ library. One of these was further characterised and appears likely to contain the DAHP synthase gene.

For my parents.

Chapter 1

Introduction

1.1 The shikimate pathway :- General introduction

In microorganisms and plants, chorismate is the common metabolic precursor of the aromatic amino acids, vitamin E and K, folic acid, ubiquinone, plastoquinone, enterochelin and various secondary metabolites. This compound is synthesised by way of the seven-step shikimate pathway (Haslam, 1974 ; Weiss and Edwards, 1980) (Figure 1.1).

Other organisms lack this biosynthetic ability and require a supplementary dietary intake of aromatic amino acids. Consequently, attention has focussed on this pathway as a potential target for pesticides and herbicides. Already, herbicides active against shikimate pathway enzymes are of great commercial importance (Grossbard and Atkinson, 1986), and in the future a greater understanding of the range of applications for plant and microbial secondary products derived from aromatic metabolism may result in a vast increase in their commercial exploitation.

Chorismate is synthesised by the action of the same seven activities in all plants and microorganisms, but the control and organisation of these activities varies radically. For example, in *E.coli* the seven steps are separable and catalysed by monofunctional proteins (Berlyn and Giles, 1969) and their genes are unlinked in the bacterial genome (Pittard and Wallace, 1966), whereas the fungal (*N.crassa*) pathway includes a pentafunctional polypeptide (the *arom* protein) which catalyses reactions two to six of the common pathway, and is the product of a single gene (Giles *et al.*, 1967 ; Lumsden and Coggins, 1977 ; Gaertner and Cole, 1977).

From the information outlined above it becomes apparent that studies on the shikimate pathway could provide information and answers pertinent to the following research topics:-

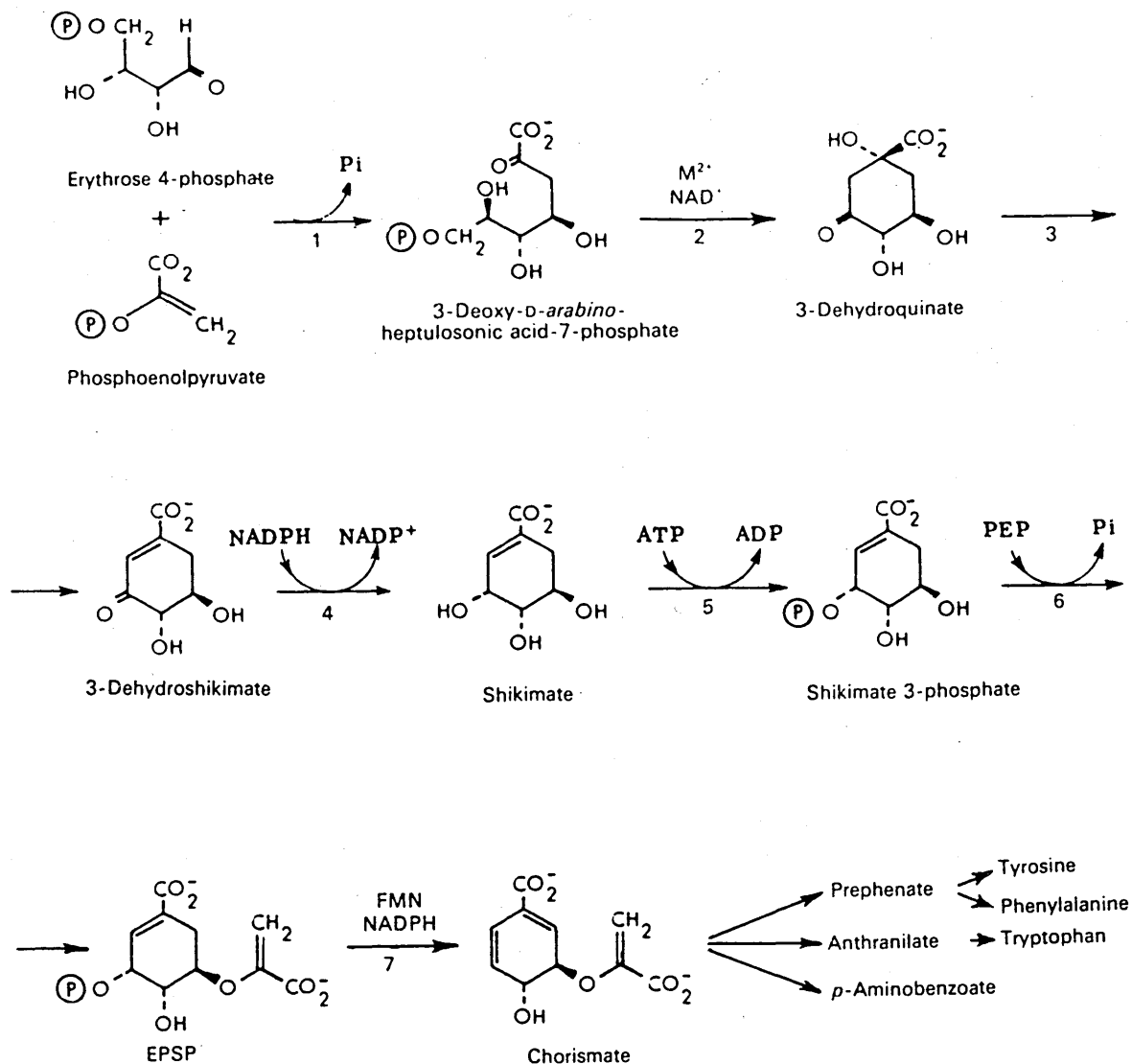


Figure 1.1 The reactions of the shikimate pathway

The numbers refer to the enzymes of the pathway: (1) 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (EC 4.1.2.15), (2) 3-dehydroquinate synthase (EC 4.6.1.3), (3) 3-dehydroquinase (EC 5.2.1.10, alternative name 3-dehydroquinate dehydratase), (4) shikimate dehydrogenase (EC 1.1.1.25), (5) shikimate kinase (EC 2.7.1.71), (6) EPSP synthase (EC 2.5.1.19, alternative name 3-phosphoshikimate 1-carboxyvinyltransferase), (7) chorismate synthase (EC 4.6.1.4). The *arom* multifunctional enzyme catalyses the reactions numbered 2–6 in the above scheme.

(a) The rational design of herbicides:- A better understanding of the chemistry of individual enzyme catalysed reactions may lead to the design of novel herbicides and pesticides, or the elucidation of the mode of action of compounds in current use.

(b) The overproduction of aromatic compounds:- An increased awareness of the control of flux through the pathway may lead to the overproduction of commercially important amino acids, or aromatic containing secondary metabolites, through the logical manipulation of the relevant biosynthetic steps.

(c) The evolution of biosynthetic pathways:- The shikimate pathway provides a model system for addressing questions on the evolution of biosynthetic pathways and multifunctional proteins in particular. Through the cloning and sequencing of genes from different organisms, and their comparison, these questions may be answered.

This introduction will describe in detail each of the reactions comprising the common pathway, with particular emphasis on the crucial first step and the mechanisms of metabolic control exercised at this point. A brief introduction to the properties of the streptomycetes will be related, before an assessment of the prospects for the improved commercial exploitation of these organisms and in particular the over-production of antibiotics by logical manipulation of relevant control mechanisms. The objectives of this thesis will then be outlined.

1.2 DAHP synthase

1.2.1 Reaction and mechanism

The oxidation of glucose via the glycolytic and pentose phosphate pathways yields

phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), respectively, which are condensed to form the phosphorylated 7-carbon keto sugar acid, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) and inorganic phosphate by the action of DAHP synthase (EC 4.1.2.15) (Srinivasan and Sprinson, 1959a) (step 1 Figure 1.1).

The mechanism of this reaction is still unclear but complementary lines of evidence indicate that it would appear not to follow the pattern of a simple aldol condensation as first proposed (Srinivasan and Sprinson, 1959b).

The simplest mechanism suggested nucleophilic attack at the phosphorous atom of PEP resulting in P-O cleavage and generation of a reactive enol pyruvate anion which can rapidly add to E4P. This explanation (Srinivasan and Sprinson, 1959b) was consistent with pyruvate kinase catalysed phosphorylations using PEP (Harrison *et al.*, 1955).

Evidence from mechanistic studies precluded this explanation. The first employing a highly purified preparation was that of De Leo *et al.*, (1973), who showed that for the *S.typhimurium* tyrosine-sensitive isozyme the reaction mechanism was ordered and sequential with PEP the first substrate to bind and DAHP the last product to leave. Subsequently other enzyme species were shown to follow the same order of addition of products and release of substrates (Simpson and Davidson, 1976 ; Schoner and Herrmann, 1976 ; Dusha and Denes, 1976 ; Sugimoto and Shiio, 1980 ; Nimmo and Coggins, 1981b).

Furthermore, De Leo and Sprinson (1968) had shown that the condensation of PEP with E4P involved C-O rather than P-O cleavage, that is when E4P reacted with PEP carrying an ^{18}O label in the C-O-P oxygen, ^{18}O was recovered in the orthophosphate released rather than in DAHP itself. A mechanism was then proposed involving initial transfer to a

nucleophilic group on the enzyme, followed by elimination of the phosphate to restore the alkene which could then initiate the aldol condensation.

Deuterium labelling experiments ruled out this enzyme intermediate possessing a freely rotating methyl group on C-3 of PEP as suggested (De Leo and Sprinson, 1968) but instead implicated an *enol*pyruvyl-enzyme intermediate with phosphate retained within the complex (Floss *et al.*, 1972).

Many DAHP synthases have been shown to be susceptible to inhibition by thiol-modifying reagents, (Staub and Denes, 1969a,b ; Gorisch and Lingens, 1971b ; Huisman and Kosuge, 1974 ; Sugimoto and Shiio, 1980 ; Ray and Bauerle, 1991) which has lead to the suggestion that sulfhydryl groups are involved either in the PEP binding site, or in the enzyme active site or in co-ordinating an essential metal co-factor.

A recent proposal about the mechanism by Ganem (1978) is consistent with the isotope labelling, product inhibition and chemical modification experiments. It proposes an enzyme-bound, sulphur-containing, *enol*pyruvyl intermediate which undergoes sulphur-migration, phosphate elimination and then participates in a nucleophilic attack on the carbon atom of the carbonyl group of E4P (Figure 1.2).

This proposal affords no role to metal ions which are believed to be an essential co-factor (Gorisch and Lingens, 1971 ; McCandliss and Herrmann, 1978 ; Nimmo and Coggins, 1980a ; Paravicini *et al.*, 1989 ; Ray and Bauerle, 1991). Chelating agents such as EDTA and *o*-phenanthroline inactivate the enzyme and the endogeneous metal has been identified as ferrous iron in several cases. Indeed, it has been suggested (Ray and Bauerle, 1991) that PEP removal facilitates metal loss from the *E.coli* tryphophan-sensitive DAHP synthase and that their mutual binding provides a stable, active enzyme. The metal may then act by stabilising the carbanion formed on C-3 of the *enol*thiopyruvate intermediate, as found for

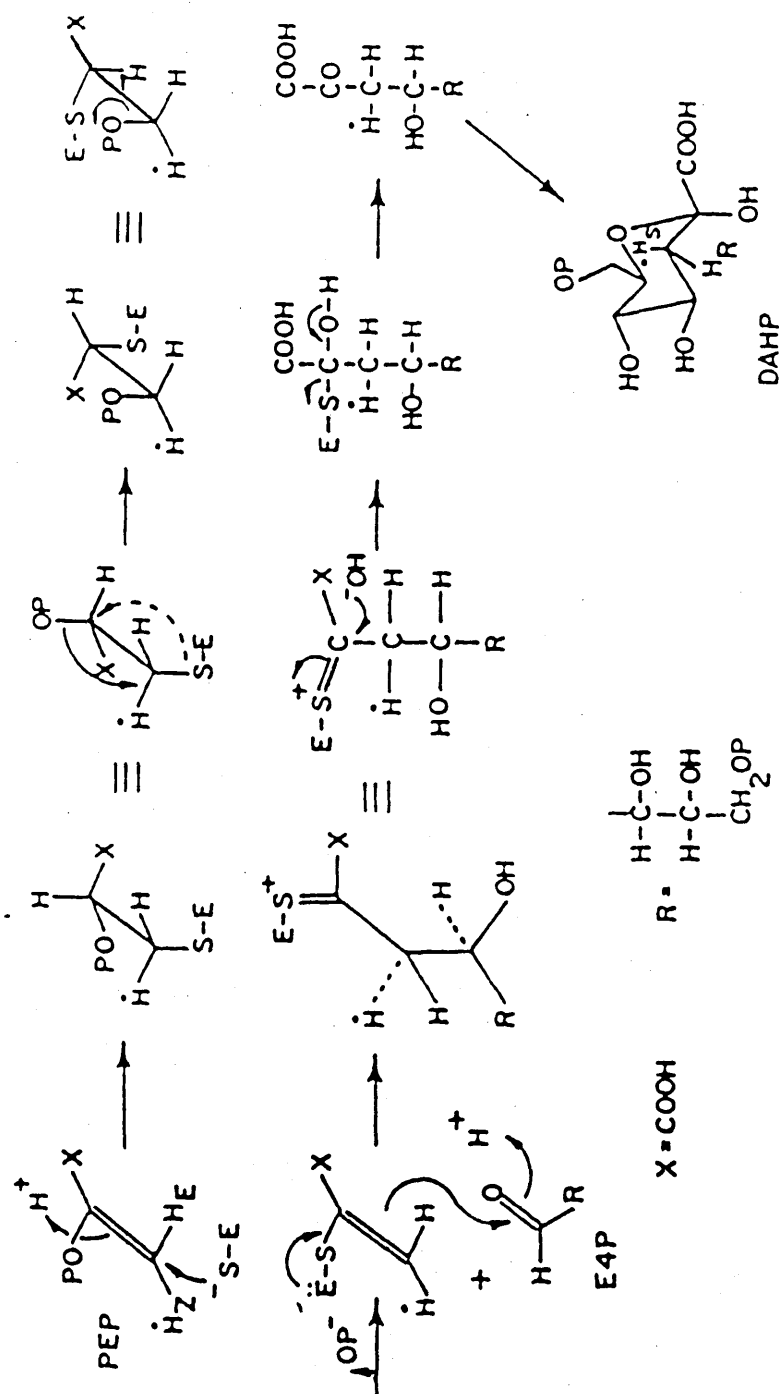


Figure 1.2 Possible reaction mechanism for DAHP synthase

The mechanism is as proposed by Ganem (1978). Conformational rearrangements are shown by the equivalence symbol, \equiv . E-S⁻ = sulfhydryl group on the enzyme.

the class II metalloaldolases (Rutter, 1964).

1.2.2 Control of activity

Branched, purely biosynthetic pathways exhibit a diversity of control mechanisms in different species. The aromatic amino acid biosynthesis pathway is no exception, with different control and organisation of the constituent enzymes well documented (Herrmann and Somerville, 1983). Carbon flow through the shikimate pathway is controlled at the first step by modulation of DAHP synthase activity either by feedback inhibition, transcriptional regulation or specific degradation.

1.2.2.1 Regulation of DAHP synthase by feedback inhibition

Diverse patterns of enzyme control have been described for DAHP synthases from different organisms (Byng *et al.*, 1982). An organism may possess from one to three isoenzymes feedback inhibited either by pathway end-products, pathway intermediates, a combination of the two or, exceptionally, the enzyme may be unregulated. Where an enzyme is responsive to more than one effector, they may act in a concerted, synergistic or cumulative manner.

The pathway end-products phenylalanine, tryptophan and tyrosine are the most common allosteric ligands but arogenate, chorismate and prephenate sensitive DAHP synthases have also been reported (Nakatsukasa and Nester, 1972 ; Ganson *et al.*, 1986 ; Ahmad *et al.*, 1986). It should be noted that some of the reported effects are of doubtful validity because of uncertainty about the quality, purity and stability of the pathway intermediates employed and whether the concentrations used reflect physiological conditions.

1.2.2.1 (a) Superfamily B prokaryotes

The best understood but probably least common regulation pattern is the three isozyme system of the enteric bacteria first described in *E.coli* (Doy and Brown, 1965). In the three isozyme system, each one is subordinated to a single end-product amino acid. In *E.coli*, the phenylalanine and tyrosine sensitive isozymes can be inhibited up to a maximum of 95% of control activity (Schoner and Herrmann, 1976 ; McCandliss *et al.*, 1978), the tryptophan isozyme is somewhat less sensitive, inhibited to a maximum of 70% of activity (Ray and Bauerle, 1991).

This pattern of regulation appears hard to rationalise with respect to the cells metabolic requirements when one considers the relative abundance of the three activities under normal conditions. In wild-type *E.coli*, 80% of the total activity is contributed by the phenylalanine-sensitive isoenzyme, 20% by the tyrosine-sensitive isoenzyme and less than 1% by the tryptophan-sensitive isoenzyme (Jensen and Nasser, 1968).

The three isoenzymes share similar kinetic properties, subunit size (Tables 3.9, 3.10) and amino acid sequence. Between the three sequences, 41% of residues are identical, and there are many clusters of identical residues. The most conserved region is a stretch between residues 52 and 190. Indeed from comparison of the first 40 *N*-terminal amino acids it was originally wrongly concluded that the isozymes had evolved independently (Poling *et al.*, 1978), since the sequence in this region contained only six conserved residues and is by far the least conserved region.

This high sequence similarity would suggest a common evolutionary origin (Pittard, 1987). However, the variations in sequence homology in different regions of the polypeptides had led to the proposal that the isoenzymes may have been constructed from the fusion of an

ancestral catalytic core with different effector binding domains (Schultz *et al.*, 1984). Definitive proof in favour of a common origin and against the domain recruitment model was provided from the results of random mutagenesis experiments on the tryptophan sensitive enzyme. This mutational analysis showed that catalytic mutants invariably occurred in conserved residue positions whereas regulatory mutants, with one exception, resulted from changes in non-conserved residues (Ray *et al.*, 1988). Both types of mutation were interspersed throughout the sequence and the feedback and catalytic regions were not physically separate and may therefore comprise overlapping sequences.

Evidence has now been gathered to support the existence of a common allosteric aromatic binding site for the isozymes which may be specifically tailored to accommodate individual amino acids (Ray *et al.*, 1988, Weaver and Herrmann, 1990).

The enteric bacteria are members of the Superfamily B group of Gram-negative prokaryotes, and a diverse pattern of control mechanisms for DAHP synthase has been described for these organisms (Jensen and Ahmad, 1988) following basic enzymic characterisation (Figure 1.3), although as yet little physical and no sequence information has been obtained. None the less, these regulatory properties have been used to delineate genealogical clusters among these Gram-negative prokaryotes which match phylogenies defined by 16S rRNA homologies and an attempt made to deduce evolutionary events which may have led from an ancestral state to the present control properties (Jensen and Ahmed, 1988) (Figure 1.3).

1.2.2.1 (b) Gram-positive bacteria

Examples from this group of bacteria possess DAHP synthases which have either a single unimetalite-responsive monofunctional isozyme or have their DAHP synthase complexed with another activity in a bifunctional polypeptide.

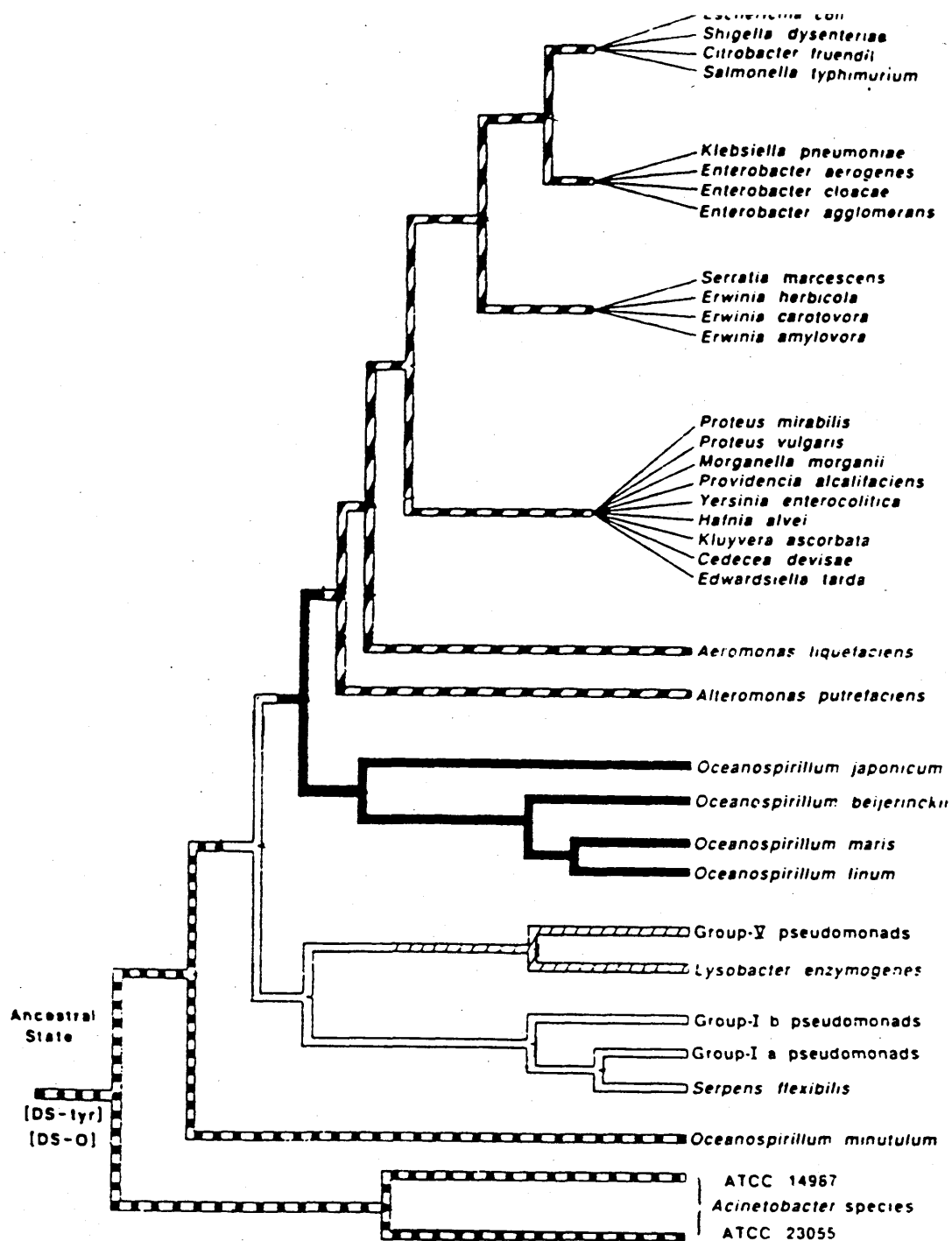


Figure 1.3 Evolution and phylogenetic distribution of DAHP synthase isoenzymes in Superfamily-B prokaryotes

The organisms have been classified according to 16S rRNA sequence data. Symbols : two isozymes, one unregulated, one tyrosine sensitive ; two isozymes, one tyrosine sensitive, one tryptophan (mainly) and chorismate sensitive ; two isozymes, one tyrosine sensitive, one tryptophan sensitive ; one enzyme, chorismate (mainly) and tryptophan sensitive ; three isozymes, one tyrosine sensitive, one tryptophan sensitive, one phenylalanine sensitive. Adapted from Jensen and Ahmed, 1982.

No evidence for the existence of isoenzymes has been reported in the *Actinomycetales* genus, with studies focussing on *Nocardia* and *Streptomyces* species (Lowe and Westlake, 1970 ; Gorisch and Lingens, 1971a ; Murphy and Katz, 1980 ; Tianhui and Chiao, 1989 ; de Boer *et al.*, 1989). At physiologically relevant concentrations the single activity was inhibited only by tryptophan.

In the *Corynebacterium*, *Brevibacterium flavum*, a single DAHP synthase was co-purified with component A of chorismate mutase, these activities residing on a 55kDa bifunctional polypeptide. A distinct polypeptide, component B, was associated and essential for chorismate mutase activity (Shiio and Sugimoto, 1979a,b). The DAHP synthase activity was synergistically inhibited by phenylalanine and tyrosine and unaffected by tryptophan (Shiio and Sugimoto, 1980). The two activities possess discrete active sites but seem likely to share a common feedback effector site (Shiio and Sugimoto, 1979a,b).

In *Bacillus subtilis* the picture is more complicated; a trifunctional complex containing DAHP synthase was described from strain 168, a pleiotropic mutant (Nester *et al.*, 1967). It consisted of a bifunctional 38 kDa protein containing DAHP synthase and chorismate mutase activities and another polypeptide carrying shikimate kinase activity (Nakatsukasa and Nester, 1972 ; Huang *et al.*, 1974). The DAHP synthase constituent was feedback inhibited only by chorismate and prephenate, the product and substrate of chorismate mutase. Later studies showed this arrangement was almost certainly due to mutation. The wild-type *Bacillus subtilis* DAHP synthase was in fact a monofunctional, prephenate inhibitable species of identical native M_r and kinetic properties to the mutant enzyme (Llewellyn *et al.*, 1980). Indeed, in the mutant a second, discrete chorismate mutase species existed which accounted for the bulk of this activity and whose purification and characteristics mirrored those of the wild-type enzyme. It was postulated that an active site capable of undertaking the chorismate mutase reaction had evolved from the allosteric

effector binding site for prephenate, and produced a second chorismate mutase activity.

1.2.2.1 (c) Lower eukaryotes

All yeast and fungi so far studied possess at least two isoenzymes inhibited by phenylalanine and tyrosine, respectively (Lingens *et al.*, 1966 ; Bode and Birnbaum, 1978 ; Fiske and Kane, 1984 ; Bode *et al.*, 1985 ; Koll *et al.*, 1988). In addition, the yeast, *Sporobolomyces salmonicolor*, and the fungus, *Neurospora crassa*, possess a third tryptophan inhibitable isoenzyme (Jensen and Nasser, 1968 ; Bode *et al.*, 1986).

Both of the *Saccharomyces cerevisiae* isoenzymes have been cloned and sequenced (Paravicini *et al.*, 1988 ; G.Braus, personal communication, 1991). The genes *ARO3* and *ARO4* encode the phenylalanine and tyrosine sensitive polypeptides, respectively, which although slightly larger are remarkably similar in sequence to the *E.coli* isoenzymes. In overlapping regions the identity with the individual *E.coli* isoenzymes can reach 53% (Figure 4.8).

Both isoenzymes are monomeric in structure and the *ARO3* protein has been purified 1250-fold from an overproducing strain where the *ARO4* gene had been deleted (Paravicini *et al.*, 1989). The enzyme is an Fe protein, and is inactivated by EDTA ; the inhibition is reversed by bivalent metal ions. The enzyme is sensitive to inhibition by phenylalanine with a K_i of 10 μ M, a value similar to that found for the *E.coli* isoenzyme.

One of the *N.crassa* isoenzymes, the least abundant tryptophan sensitive species, has been purified to homogeneity (Nimmo and Coggins, 1981a). It comprises 14% of the total enzyme activity compared to 54% and 32% provided by the tyrosine and phenylalanine sensitive isoenzymes, respectively (Nimmo and Coggins, 1981a). It had a larger subunit

Mr, 52kDa, was tetrameric and EDTA sensitive. No sequence data is yet available.

1.2.2.1 (d) Plants

The control of the shikimate pathway in plants may satisfy different criteria from those in microorganisms. The quantitative importance of the pathway in plants is much greater, providing as it does, in addition to vitamins and amino acid requirements, secondary products, lignins and other aromatics. Additionally the pathway may be regulated in a tissue-specific manner or developmentally. Finally the location of the pathway within a cell may be plastidic or cytosolic (Jensen, 1986).

Present evidence indicates the existence of at least two isozymic forms of DAHP synthase as found in a variety of higher plants (Rubin *et al.*, 1982 ; Rubin and Jensen, 1985 ; Ganson *et al.*, 1986 ; Morris *et al.*, 1989). They differ in their metal ion requirements (being Mn and Co dependant, respectively), pH optimum, substrate kinetics, susceptibility to inhibition by DTT, and subcellular location (the Mn isozyme is believed to be plastidic, the Co isozyme cytosolic). The plant enzyme followed an orthodox pattern of inactivation by chelating agents and sulfhydryl modifying reagents (Huisman and Kosuge, 1974).

A third isozyme has been resolved in pea extracts (Rothe *et al.*, 1976) and carrot cell extracts (Suzich *et al.*, 1985), but there is the suggestion that this could represent another sugar aldolase capable of mimicking the DAHP synthase reaction (Morris *et al.*, 1989).

The pattern of response to allosteric effectors is as varied as found in any other group of organisms. In one case, that of *Cinchona succirubra*, the enzyme was responsive to inhibition by phenylalanine, tyrosine and tryptophan (Schmauder *et al.*, 1985), but more often a completely feedback resistant isozyme has been found, such as those described from cauliflower (Huisman and Kosuge, 1974, *Camellia sinensis* (Saijo and Takeo, 1979)

and maize root (Graziana and Boudet, 1980). Contrastingly, in an example of differential tissue-specific regulation, enzyme prepared from maize shoot was inhibited by tryptophan (Graziana and Boudet, 1980). A more complex pattern has been described for the Mn-dependant isozyme from mung bean. Prephenate, arogenate, and to a lesser extent tryptophan, inhibited this isozyme, whereas chorismate enhanced the activity. The separable Co-dependant activity was inhibited solely by the secondary metabolite, caffeic acid. This pattern was rationalised as a sequential feedback inhibition mechanism, controlling the entire flux through the pathway (Rubin and Jensen, 1985).

Other enzyme species from carrot and potato tubers were insensitive to feedback inhibition and instead are activated by pathway end-products tryptophan (mainly) and tyrosine (to a lesser extent) (Suzich *et al.*, 1985 ; Pinto *et al.*, 1986). This feedback activation may be a response to wounding and promote the production of lignins for callous formation (Suzich *et al.*, 1985).

The two plant DAHP synthases which have been purified to homogeneity from carrot and potato are dimers of apparent subunit M_r of 53kDa (Suzich *et al.*, 1985 ; Pinto *et al.*, 1986). A cDNA encoding the potato enzyme has been cloned, and the deduced amino acid sequence indicates a polypeptide of M_r 56,153 (Dyer *et al.*, 1990). cDNA/RNA hybridisations and antibody cross-reactivities indicate the close sequence and structural similarities of a range of plant DAHP synthases across diverse evolutionary distant species (Dyer *et al.*, 1990). Overall sequence identity between the plant and bacterial enzymes is less than 25%, and the plant enzyme possesses an additional 140 amino acid residues at the carboxy terminus whose function is not known (Dyer *et al.*, 1990).

A goal of future work must be to clone and sequence different plant isozymes, compare them, and identify individual residues important in conferring the different kinetic

properties and effector sensitivity.

1.2.3 Transcriptional regulation of DAHP synthase

1.2.3 (a) Superfamily B prokaryotes

The isozymes of enteric bacteria, alone among this group, have been shown to possess repression/depression control by the corresponding amino acids (Wallace and Pittard, 1967 ; Jensen and Nasser, 1968 ; Ahmad *et al.*, 1987).

In *E.coli*, the structural genes *aroF*, *aroG*, and *aroH* encode the tyrosine-sensitive, phenylalanine-sensitive and tryptophan-sensitive isozymes, respectively. The control of expression of the genes is exercised at the transcriptional rather than the translational stage, and with the possible exception of *aroF*, this control is mediated solely by repressor proteins with product amino acids acting as corepressors.

The expression of *aroH* is controlled by tryptophan mediated through *trpR* (Pittard *et al.*, 1969). Repression alone is thought to be the method of transcriptional control as the DNA sequence of the monocistronic operon precludes attenuation control (Zurawski *et al.*, 1981). The variation in *aroH* expression found has not been reported, possibly due to the very low levels of transcript which would exist even under de-repressed conditions.

The transcription of both the *aroF* and *aroG* genes is controlled by the *tyrR* gene product (Wallace and Pittard, 1969 ; Brown and Somerville, 1971 ; Im *et al.*, 1971 ; Mattern and Pittard, 1971 ; Camakaris and Pittard, 1973 ; Im and Pittard, 1973). The TyrR protein acts on a specific operator sequence, a 17 nucleotide long palindrome known as the TYR box (DeFeyter *et al.*, 1986). Eight genes are under the control of the TyrR protein, they possess at least a single TYR box at the operator loci and are known as the *tyrR* regulon (Pittard, 1987). The expression of *aroG* is co-repressed by phenylalanine and tryptophan,

with this varying over a two-fold range and not fully repressible. *aroF* and *tyrA*, the structural gene for chorismate mutase-prephenate dehydrogenase, form the *tyr* operon under the control of tyrosine mediated by *tyrR* (Gollub and Sprinson, 1969 ; Mattern and Pittard, 1971). The expression can vary over 100 fold and can be repressed completely. In addition, *aroF* expression may be under some form of attenuation control (Camakaris *et al.*, 1980, Hudson and Davidson, 1984)

Therefore, the various operators associated with the genes of the *tyrR* regulon differ in their ability to bind the corepressor forms of the TyrR protein (eg. repressor-tyrosine, repressor phenylalanine). These phenomena can be explained with respect to the quantity and sequence of the TYR boxes, as TyrR protein molecules can bind cooperatively at adjacent boxes and some box sequences bind TyrR protein more avidly.

In addition to these control mechanisms, under starvation conditions the tyrosine isozyme is specifically degraded (DeLucia *et al.*, 1979 ; Tribe and Pittard, 1979) with the *tyrA* product unaffected (DeLucia and Herrmann, 1983).

Although the repressor control systems play an important role in regulating pre-formed enzyme levels, it is the control of intact enzyme by feedback inhibition which appears the quantitatively major regulatory mechanism of flux through the common pathway *in vivo* (Ogino *et al.*, 1982).

1.2.3 (b) Gram-positive bacteria

No convincing evidence for repression control of the expression of shikimate pathway genes has been reported in the genus *Actinomycetales* (discussed in section 3.13).

In *Brevibacterium flavum*, the synthesis of both polypeptides comprising the DAHP synthase/chorismate mutase bifunctional complex is subject to regulation by tyrosine (Shiio and Sugimoto, 1979b). Tyrosine is also involved in the mediation of transcriptional control of the *Bacillus subtilis* DAHP synthase (Nester *et al.*, 1969) with biosynthesis of the aromatic amino acids and histidine perhaps sharing mutual regulatory elements (Chapman and Nester, 1968).

1.2.3 (c) Lower eukaryotes

In *Saccharomyces cerevisiae* *ARO3* and *ARO4* are under the control of at least two promoters (Paravicini *et al.*, 1989 ; G.Braus, personal communication, 1991) recognised by different transcriptional factors whose binding is mutually exclusive. Basal expression of the genes is amino acid independent, but under starvation conditions the genes are derepressed to a higher transcription rate by GCN4, the general control activator protein, acting at the second promoter site. This mechanism may facilitate cell growth in the presence of tyrosine and phenylalanine, when tryptophan would still be required.

No other transcriptional control mechanisms have yet been reported from other organisms in this group with respect to DAHP synthase.

1.2.3 (d) Plants

Levels of DAHP synthase in plants vary during development and in response to environmental influences. The specific activity of the enzyme increases during seed germination of *Vigna radiata* (Minamikawa, 1967), *Pisum sativum* (Rothe *et al.*, 1976), *Zea mays* (Graziana and Boudet, 1980) and during shoot formation from *Nicotinia tobacum* (Beaudoin-Egan and Thorpe, 1983).

Potato DAHP synthase activity is elevated in response to stress by the herbicide glyphosate by *de novo* synthesis of the plastidic enzyme (Pinto *et al.*, 1988). Glyphosate inhibits EPSP synthase, and the signal for DAHP synthase induction may be the depletion of aromatic amino acids or secondary products. Wounding potato or tomato tissue also induces synthesis of DAHP synthase (Dyer *et al.*, 1989) as does treatment of parsley cells with a fungal elicitor (McCue and Conn, 1989). Finally, light treatment of parsley cells increases levels of the plastidic isozyme, whilst the cytosolic species is unaffected.

The control elements which may lie upstream of the DAHP synthase protein coding region remain uninvestigated, and their elucidation may provide the answer to questions about transcriptional control mechanisms and implicate the protein factors involved.

1.3 3-Dehydroquinate synthase

The second reaction of the shikimate pathway, namely the conversion of DAHP to DHQ, is catalysed by 3-dehydroquinate synthase (EC 4.6.1.3) (step 2 Figure 1.1). This reaction was first demonstrated in 1963 (Srinivasan *et al.*, 1963), following the earlier isolation and identification of DHQ as a pathway intermediate from the culture supernatant of aromatic auxotrophs of *E.coli* (Weiss *et al.*, 1953).

Following experiments involving substituted analogues and radiolabelled substrates, the reaction was shown to involve an intramolecular exchange of the DAHP ring oxygen with C-7, accompanied by an oxidation at C-6 and a reduction at C-2 (LeMarechal and Azerad, 1976 ; Rotenberg and Sprinson, 1970, 1978). The driving force for the reaction is the cleavage of the phosphoester. It was shown that both NAD^+ and a divalent metal were essential for activity, with a crucial step in the overall conversion being the oxidation and then reduction of the C-5 of DAHP involving NAD^+ (Srinivasan *et al.*, 1963). The

enzyme is believed to recognise the pyranose form of DAHP (Lambert *et al.*, 1985).

A monomeric protein of 40 kDa M_r catalyses the reaction in *E.coli*, with a K_m for DAHP of 33 μ M (Maitra and Sprinson, 1978 ; Frost *et al.*, 1984). In *Bacillus subtilis* the enzyme has a M_r of 17 kDa, a K_m for DAHP of 130 μ M, and is only active when associated with chorismate synthase and a flavin reductase in a trifunctional complex.

In *E.coli*, DHQ synthase appears to be synthesised constitutively, and the activity found in wild-type extracts is calculated to be about five-fold greater than would be required to satisfy the aromatic requirements of vigorously growing cells (Tribe *et al.*, 1976). In strains of *E.coli* with a feedback-resistant DAHP synthase (tyrosine), DHQ synthase activity becomes rate-limiting for flux through the pathway with consequent accumulation of DAHP (Ogino *et al.*, 1982).

1.4 3-Dehydroquinase

The enzyme 3-dehydroquinase (EC 4.2.1.10) catalyses the third step of the shikimate pathway, namely the stereospecific (*syn*) elimination of water to yield 3-dehydroshikimate (step 3 Figure 1.1). This compound contains the first double bond of the aromatic ring system (Salamon and Davis, 1953 ; Srinivasan *et al.*, 1956 ; Hanson and Rose, 1963 ; Turner *et al.*, 1975).

The reaction proceeds through the formation of a Schiff base between a lysyl side chain amino group and the keto group of 3-dehydroquinone (Butler *et al.*, 1974 ; Chaudhuri *et al.*, 1991). This causes a conformational change in the substrate, thereby facilitating the stereospecific course of the reaction (Vaz *et al.*, 1975). A basic group, shown to be the imidazole side chain of a histidine residue participates in proton abstraction (Walsh, 1979 ;

Chaudhuri *et al.*, 1986).

Four types of enzyme have been described. Three comprise the biosynthetic 3-dehydroquinases, found in bacteria, plants, yeast and fungi whereas the fourth type is found only in fungi. The fourth type, the catabolic dehydroquinases, which are found only in fungi such as *N.crassa* and *A.nidulans*, are induced in response to quinic acid and are involved in the utilisation of quinic acid as carbon source for growth (Giles *et al.*, 1985). The biosynthetic enzymes are either dimers of subunit size 27,000 (Chaudhuri *et al.*, 1986) or form a domain within a multifunctional protein corresponding to this size (Duncan *et al.*, 1987 ; Mousedale *et al.*, 1987). The catabolic enzymes are large multimeric proteins (probably dodecamers) consisting of identical monofunctional subunits of M_r 16,000-18,000 (Hawkins *et al.*, 1982 ; Da Silva *et al.*, 1986).

There are two sub-types of multifunctional enzyme containing 3-dehydroquinase. In plants, shikimate dehydrogenase shares a single bifunctional polypeptide with the 3-dehydroquinase activity (Polley, 1978 ; Koshiba, 1978 ; Mousedale *et al.*, 1987) while in fungi and yeast 3-dehydroquinase is one of the five catalytic components of the *arom* multifunctional enzyme (Lumsden and Coggins, 1977 ; Gaertner and Cole, 1977). When the sequences of the *E.coli* and fungal enzymes were compared they were found to be similar at the primary sequence level (Duncan *et al.*, 1987).

Curiously, the biosynthetic 3-dehydroquinase of *S.coelicolor* displays physical and kinetic properties, and sequence similarities with the fourth type of 3-dehydroquinases the inducible catabolic type (White *et al.*, 1990). These inducible enzymes show little similarity to the biosynthetic dehydroquinases at the primary sequence level (Duncan *et al.*, 1987) and do not appear to catalyse the reaction via a Schiff base mechanism, as attempts to inactivate enzyme from *S.coelicolor* and *A.nidulans* with $NaBH_4$ have failed (Walker, G.E., unpublished, 1990 ; Kleanthous, K., unpublished, 1990).

1.5 Shikimate dehydrogenase

Shikimate dehydrogenase (EC 1.1.1.25) converts 3-dehydroshikimate to shikimate (Yaniv and Gilvarg, 1955) utilizing NADPH as proton donor in a stereospecific manner (Dansette and Azerad, 1974) (step 4 Figure 1.1).

In *E.coli*, the enzyme is a monomer of 32,000 M_r (Chaudhuri and Coggins, 1985) and the derived sequence contains a consensus nucleotide binding region which probably represents the NADPH binding site (Anton and Coggins, 1988). As mentioned previously (section 1.4), the activity resides with 3-dehydroquinase on a bifunctional protein in a variety of plants and on the *arom* multifunctional protein in yeast and fungi (Lumsden and Coggins, 1977 ; Hawkins, 1987 ; Duncan *et al.*, 1987).

1.6 Shikimate kinase

Shikimate kinase (EC 2.7.1.71) catalyses the transfer of phosphate from ATP to the C-3 -OH group of shikimate to form shikimate 3-phosphate (Weiss and Mingioli, 1956) (step 5 Figure 1.1).

No aromatic auxotrophs blocked in this reaction could be found in *E.coli* (Pittard and Wallace, 1966a) or *S.typhimurium* (Gollub *et al.*, 1967) and it was established that both possessed isoenzyme forms (Morrell and Sprinson, 1968 ; Berlyn and Giles, 1969). While expression of shikimate kinase of *S.typhimurium* appears to be unregulated (Gollub *et al.*, 1967) and the shikimate kinase I of *E.coli* is expressed constitutively, the second isozyme, shikimate kinase II, encoded by *aroL*, is under the specific control of the *tyrR* regulator gene, its synthesis being repressed by tyrosine or tryptophan.

The existence of isofunctional enzymes frequently occurs in the first reaction of a pathway that later branches to different end-products (eg. DAHP synthase), furthermore specific modulation of gene expression usually affects genes encoding the first enzyme in a pathway and in the example of the shikimate pathway in *E.coli* only the three DAHP synthases and shikimate kinase II are subject to such control. This has led to the hypothesis that an unknown metabolic pathway branches from the shikimate pathway at this point or that in the evolutionary past, aromatic biosynthesis originated at this point (Pittard, 1987).

However, an examination of the kinetic properties of the *E.coli* isozymes indicated that shikimate kinase I may possess another function and only fortuitously catalyse this reaction. Shikimate kinase II has been cloned, sequenced and overexpressed and is a monomer of 20,000 M_r (DeFeyter *et al.*, 1986 ; DeFeyter and Pittard, 1986 ; Millar *et al.*, 1986). The K_m of shikimate kinase II for shikimate is 200 μM, whereas that of shikimate kinase I is in excess of 5 mM (cited in Pittard, 1987). Furthermore, the two genes would appear not to be homologous (cited in Pittard, 1987). The true function then for shikimate kinase I remains unknown.

In yeast and fungi shikimate kinase occurs on the *arom* multifunctional polypeptide (Lumsden and Coggins, 1977 ; Hawkins, 1987 ; Duncan *et al.*, 1987).

1.7 EPSP synthase

Shikimate 3-phosphate and phosphoenolpyruvate react to form 5-enolpyruvyl-shikimate 3-phosphate (EPSP) and inorganic phosphate via the action of EPSP synthase (EC 2.5.1.19) (Levin and Sprinson, 1964) (step 6 Figure 1.1). This enzyme has arguably been studied more exhaustively than any other in the pathway in terms of mechanism and structure, for the good reason that this activity is the major target for inhibition by the broad

spectrum herbicide, glyphosate (Steinrucken and Amrhein, 1980 ; Boocock and Coggins, 1983), which is of considerable economic importance (Grossbard and Atkinson, 1986).

The mechanism involves the protonation of C-3 of PEP and the transfer of the *enol*pyruvate moiety of PEP onto the hydroxyl group at C-5 on the acceptor molecule, shikimate 3-phosphate, to form a tetrahedral intermediate. The cleavage of the C-O bond at C-2 of PEP facilitates the elimination of the phosphate (Levin and Sprinson, 1964 ; Bondinell *et al.*, 1971). This mechanism first proposed in 1964 has recently been conclusively validated by the isolation of the predicted tetrahedral intermediate (Anderson *et al.*, 1988a, b).

A number of amino acids have been implicated in catalysis including arginine, cysteine, glutamate, histidine and lysine residues (reviewed in Bentley, 1990). The role of these residues will become more apparent when a high resolution three-dimensional structure of the enzyme is available. Crystals diffracting to give medium-resolution are now available (Abdel-Meguid *et al.*, 1985).

EPSP synthase from *E.coli* has been purified, sequenced and overexpressed (Lewendon and Coggins, 1983 ; Duncan *et al.*, 1984 ; Duncan *et al.*, 1985) and is a monomer of 46,112 M_r. The crucial step in the purification from wild-type *E.coli* was substrate elution from phosphocellulose. Shikimate 3-phosphate and PEP together were the most effective in eluting the activity and indicate an ordered reaction in which shikimate 3-phosphate binds to the enzyme first.

This purification was followed by others from a range of bacteria and plants, including *S.typhimurium* (Stalker *et al.*, 1985), *K.pneumoniae* (Steinrucken and Amrhein, 1984) and pea seedling (Mousedale and Coggins, 1984), with sequence data also being obtained

for the first two. Subsequently other sequences were obtained from *Bordetella pertussis* (Maskell *et al.*, 1988), petunia (Gasser *et al.*, 1988), pea (Granger, 1989), tomato and *Arabidopsis thaliana* (Klee *et al.*, 1987), yeast (Duncan *et al.*, 1987), and *Aspergillus nidulans* (Charles *et al.*, 1986). There is considerable homology between the plant, bacterial and fungal sequences.

As mentioned above, EPSP synthase is the site of action of glyphosate but despite a large amount of research the precise nature of this effect is not clear. It was shown for the EPSP synthase component of the *N.crassa arom* complex that the reaction proceeds sequentially with shikimate 3-phosphate (S3P) the first substrate to bind followed by PEP. The enzyme was not able to bind PEP nor glyphosate in the absence of S3P. The inhibitory action of glyphosate results from its competition with PEP to bind the enzyme-S3P complex (Boocock and Coggins, 1983). The ionic form of glyphosate may act as a transition state analogue of PEP (Amrhein, 1986) and recent studies utilizing NMR chemical shift perturbations have elucidated the conformation of glyphosate bound within the enzyme dead-end complex (Castellino *et al.*, 1989). Indeed, more potent EPSP synthase inhibitors, phosphonate analogues of the tetrahedral reaction intermediate have now been synthesised (Alberg and Bartlett, 1989).

1.8 Chorismate synthase

Chorismate synthase (EC 4.6.1.4) catalyses the seventh and last reaction of the shikimate pathway, namely the *trans* 1,4-elimination of orthophosphate from EPSP (Hill and Newkome, 1969 ; Onderka and Floss, 1969 ; Floss *et al.*, 1972) to yield chorismate (Gibson and Jackman, 1963 ; Gibson and Gibson, 1964) (step 7 Figure 1.1). The enzyme is characterised by an absolute requirement for a reduced flavin cofactor to carry out the reaction, but this cofactor is not stoichiometrically consumed during the reaction and its role remains ambiguous.

The complete sequence of the *E.coli* enzyme has been determined and the enzyme purified from an overproducing strain (White *et al.*, 1988). It is a tetramer of 38,000 M_r, whereas the enzyme from *N.crassa* is larger, comprising a tetramer of 50,000 M_r (White *et al.*, 1988).

The *N.crassa* chorismate synthase has the ability to reduce FMN in the presence of NADPH via an intrinsic flavin reductase (diaphorase) activity (Welch *et al.*, 1974), indicating that each polypeptide of the homotetramer is bifunctional, possessing both chorismate synthase and diaphorase activity. The *E.coli* enzyme does not possess this intrinsic diaphorase activity and can only catalyse the formation of chorismate when supplied with exogenous reduced flavin (Morrel *et al.*, 1967 ; White *et al.*, 1987). It has been proposed that the *N.crassa* polypeptide consists of two domains, one catalysing the formation of chorismate from EPSP and one carrying diaphorase activity and responsible for providing reduced flavin cofactor (White *et al.*, 1988). The smaller subunit *E.coli* enzyme may consist of a single catalytic activity and lack a diaphorase domain.

A monofunctional enzyme has been characterised from pea (Mousedale and Coggins, 1986) and appears to be analogous to the *E.coli* type. A bifunctional enzyme complex was found in *Bacillus subtilis* (Hasan and Nester, 1978) ; in this case a monofunctional chorismate synthase polypeptide was associated with a 13,000 M_r NADPH -dependent flavin reductase. The M_r of this flavin reductase component is very similar to the size difference between the *E.coli* and *N.crassa* enzymes.

The genus *Streptomyces* comprises a group of gram-positive obligately aerobic bacteria characterised by their morphological and metabolic capabilities. They inhabit soils, and as such derive much of their nutrition from insoluble organic detritus, such as leaves. The ability to produce and secrete extracellular hydrolytic enzymes such as cellulases, amylases, proteases and nucleases facilitates the digestion of insoluble materials to products suitable for uptake into the cell (Williams *et al.*, 1983).

The morphology and life-style of the organism reflect this adaptation. They grow as a mycelium of branching hyphae able to penetrate substrate locations. The colony can secrete and produce a high local concentration of degradative enzymes, and as the hyphae within a colony are inter-connected, nutrients obtained at one location can be shared with nutrient-deficient regions of the colony.

The sedentary, mycelial habit exploits this ecological niche efficiently but upon nutrient limitation a mechanism is required to allow dispersal to a new environment. This is achieved by a reproductive phase following the termination of vegetative colonial growth. At this stage, the substrate mycelium gives rise to differentiated spore-bearing aerial hyphae which derive nutrients from the lysing substrate mycelium and antibiotics are also produced (Figure 1.4). The role of antibiotics may be to protect the producer organism at vulnerable stages of its life-cycle from motile microbes which may compete for the use of lysed substrate mycelia (Chater, 1984).

The antibiotics produced by streptomycetes are of considerable interest as a result of their medical, veterinary and agricultural importance (Berdy, 1980). Of the thousands of naturally occurring antibiotics discovered so far, about two-thirds including many of great

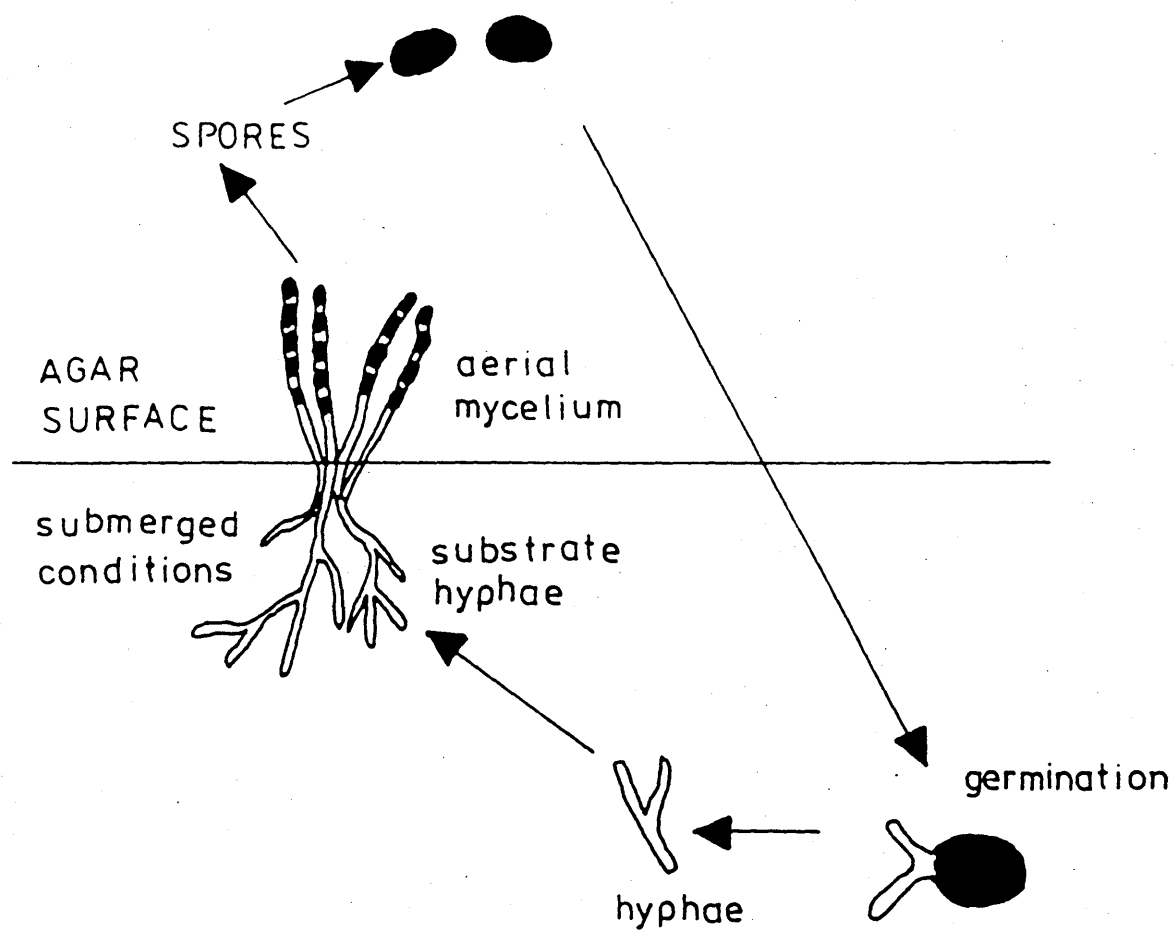


Figure 1.4 Schematic representation of the life-cycle of streptomycetes

commercial importance are produced by streptomycetes and their relatives in the actinomycetes. Much research has therefore focussed on the biosynthesis of antibiotics and on the improvement of antibiotic yield from these organisms.

1.9.1 The choice of *Streptomyces coelicolor*

The organism of choice for our study on the shikimate pathway in streptomycetes was *Streptomyces coelicolor*. It is genetically the most extensively characterised streptomycete species, having been studied by both classical and molecular approaches (Hopwood, 1988). The opportunity to manipulate genes and regulatory mechanisms has been made possible through the development of host-vector systems for gene cloning (Hopwood *et al.*, 1985). The availability of a chemically defined medium which permitted dispersed growth (Hobbs *et al.*, 1989) was another important criterion. Finally, the fact that no commercially important products are produced by *Streptomyces coelicolor* ensures that there is a full range of literature published on disparate aspects of physiology, genetics and biochemistry of this essentially academic strain.

1.9.2 The secondary products of *Streptomyces coelicolor*

A secondary product is defined as a substance that occurs in a limited number of organisms, not universally, and the function of which, if known, is not that of an intermediate in metabolism (Weiss and Edwards, 1980). In plants, these products may be involved in product storage, detoxification and pigmentation (Weiss and Edwards, 1980). In streptomycetes, the association of the production of secondary metabolites with morphological differentiation alludes to the role of these products as either regulators of the process or as protectors of the producer organism during this process (reviewed in Chater, 1989).

Streptomyces coelicolor produces at least five secondary products, three of which, actinorhodin, undecylprodigiosin and methylenomycin, have been extensively characterised (reviewed in Hopwood, 1988). Actinorhodin is a diffusible blue pigmented polyketide product, chromosomally encoded (Wright and Hopwood, 1976), the production of which is sensitive to inhibition by ammonia and to a lesser extent by phosphate, and is not growth associated (Hobbs *et al.*, 1990). Undecylprodigiosin is also pigmented and chromosomally encoded (Rudd and Hopwood, 1980) being red in colour (Tsao *et al.*, 1985) and mycelially associated (Rudd and Hopwood, 1980). It is produced toward the end of exponential growth (Walker G.E, unpublished results, 1988) and the regulation of production differs from actinorhodin in being only slightly affected by ammonia and phosphate (Hobbs *et al.*, 1990). The third identified product, methylenomycin, an epoxycyclopentanone, is colourless, diffusible, plasmid encoded (a conjugative plasmid termed SCP1) (Kirby and Hopwood, 1977), with its mode of action and biosynthesis still unknown.

The sets of genes encoding the biosynthetic steps of these compounds have been cloned and analysed (Malpartida and Hopwood, 1984 ; Chater and Bruton, 1985 ; Malpartida *et al.*, 1990).

None of the secondary products so far described from *Streptomyces coelicolor* contain chemical groups originating from the shikimate pathway.

1.10 Antibiotic biosynthesis in streptomycetes

As stated above, about two-thirds of the naturally occurring antibiotics presently identified are produced by streptomycetes and their relatives, the other significant producers being *Bacillus* sp., *Myxobacterium* sp. and mycelial fungi. Furthermore, these diverse producer

organisms share the same characteristics of soil-habitat and spore formation.

Antibiotics are classified according to chemical structure and their inhibitory mode of action against target micro-organisms. Their specificity of action has made them invaluable in the fields of medicine and agriculture and as research tools in biochemistry. However, these structures arise from simple precursors derived from intermediary metabolism. Acetate, propionate, glucose, and amino acids are precursors for complex multi-step pathways. The final bio-active product may be synthesised either by the combination of separate precursor components, or the simple step-wise modification of a single molecule.

Antibiotic biosynthesis is exquisitely regulated and this regulation must be interpreted within an overall understanding of the differentiation process and life-cycle of the organism.

1.10.1 Organisation and regulation

By the nature of their biocidal activity and because of the metabolic expense of their production the biosynthesis of antibiotics must be tightly regulated.

It has been found that antibiotic synthesis is switched on late in growth, often being sensitive to repression by particular growth nutrients, including metabolisable carbon sources, nitrogen (usually in the form of ammonia), and inorganic phosphate (Martin and Demain, 1980). Some other pathways are activated by hormone-like substances, for example the A-factor of *S.griseus*, *S.bikiniensis* and *S.coelicolor*, diffusing within a colony. These substances are also essential for normal spore development (Kokhlov *et al.*, 1973, Hara *et al.*, 1983). Hence, some regulatory mechanisms link metabolic change to morphological differentiation.

Producer organisms possess resistance mechanisms to protect themselves against the toxic effects of their own metabolites. Thus, if the mode of action of an antibiotic was anti-ribosomal (such as erythromycin), then the producer organism has the ability to modify and render unaffected its own ribosomal RNA. Other resistance mechanisms include intracellular inactivation eg. bialaphos (Murakami *et al.*, 1986), or active export eg. methylenomycin (Neal and Chater, 1987). Some organisms possess more than one resistance determinant, and speculation surrounds this feature. In the case where a complex antibiotic is composed of two or more independently synthesized components then a resistance mechanism may reside with the set of biosynthesis genes for each component (Chater and Hopwood, 1989).

There is thus a close relationship between the genes encoding antibiotic resistance and the genes encoding biosynthetic enzymes. It is now accepted that the genes for the biosynthesis of most antibiotics are encoded by clusters of genes on the chromosome which contain regulatory elements and resistance genes. Indeed the strategy of cloning resistance genes has enabled the cloning of entire biosynthetic pathways which flank these determinants (eg. Murakami *et al.*, 1983).

The clustering of genes also raises the possibility of transferring the potential to produce one antibiotic from one organism to another and this has now been done several times (eg. Malpartida *et al.*, 1990). Furthermore, the production of hybrid antibiotics by the action of enzymes from different species within another is possible (Hopwood *et al.*, 1985 ; Omura *et al.*, 1986).

Transcriptional regulation within antibiotic clusters is complex. Multiple promoter regions and divergent directional transcription combine to facilitate complex transcriptional control. It is clear that there is transcriptional interplay between biosynthetic and resistance genes to

ensure an organism is resistant before it embarks on production of the lethal metabolite. Multiple promoters recognised by RNA polymerases with distinct sigma factors may also be important in specific gene expression during differentiation and antibiotic production (Westpheling *et al.*, 1985 ; Buttner *et al.*, 1988).

1.10.2 Structural classes of antibiotics, their modes of action and their applications

The three chief starting materials for secondary metabolism are :-

- (1) the shikimate pathway products, which are incorporated into several classes of antibiotics.
- (2) other amino acids, which form components of peptide antibiotics, peptidolactones, penicillins and cephalosporins.
- (3) acetate, the major constituent of the polyketides which include macrolide, polyene, ansamycin, tetracycline and anthracycline antibiotics.

Antibiotic structures may be composed solely from one of these groups or incorporate chemical groups from more than one.

1.10.2 (a) β -lactams

Among the β -lactam antibiotics, penicillins and cephalosporins are clinically the most valuable. Penicillin (Figure 1.5) is biosynthesised from the precursors L- α -aminoadipic acid, L-cysteine and L-valine; cephalosporins are derived from penicillins following further modification (Figure 1.6). The majority of antibiotics of this class are produced by streptomycetes, with a significant proportion contributed by mycelial fungi such as *Penicillium* sp. and *Cephalosporium* sp. (O'Sullivan and Ball, 1983).

The anti-microbial activity of penicillins is a consequence of their ability to inhibit bacterial

cell wall synthesis. They form a covalent ternary complex with the bacterial transpeptidase enzyme, hence irreversibly inactivating the enzyme.

1.10.2 (b) Peptides

Peptide antibiotics produced by *Bacillus* species are synthesised from both the L-amino acids found in proteins and from unusual amino acids, such as D-isomers and *N*-methyl derivatives. The process involves activation of the substrate amino acid, enzyme aminoacylation, amino acid racemization and peptide bond formation. These steps are carried out by large, multifunctional proteins using a "thiotemplate" (Kleinkauf and von Doren, 1983).

1.10.2 (c) Peptidolactones

Most peptidolactones are produced by the actinomycetes with a small minority produced by *Bacillus* species. These antibiotics consist of two parts :- a peptide chain, which incorporate unusual amino acids (see 1.10.2 (b)), and an acyl group. The two are joined together at the *N*-terminal amino group of the peptide.

An example is the actinomycins, composed of a phenoxazinone chromophore and two pentapeptide chains (Figure 1.7). The sequence of the amino acids in the peptide chain differs between individual actinomycins. The chromophore unit is derived from tryptophan.

Actinomycins are biologically active against gram-positive bacteria but considerably less so against gram-negative bacteria and fungi. They also have been used in the treatment of cancers (Okumura, 1983).

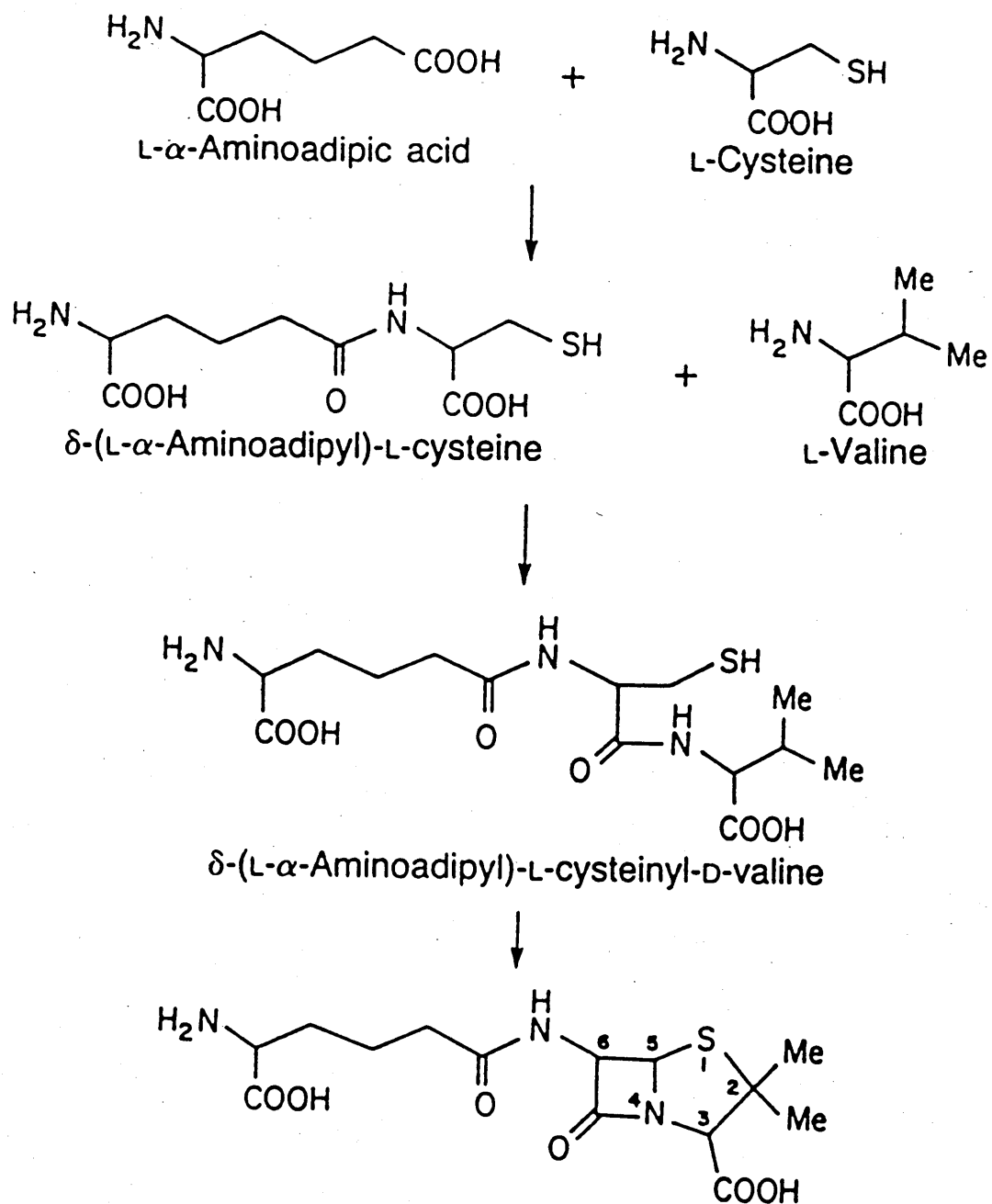


Figure 1.5 Biosynthesis of isopenicillin N

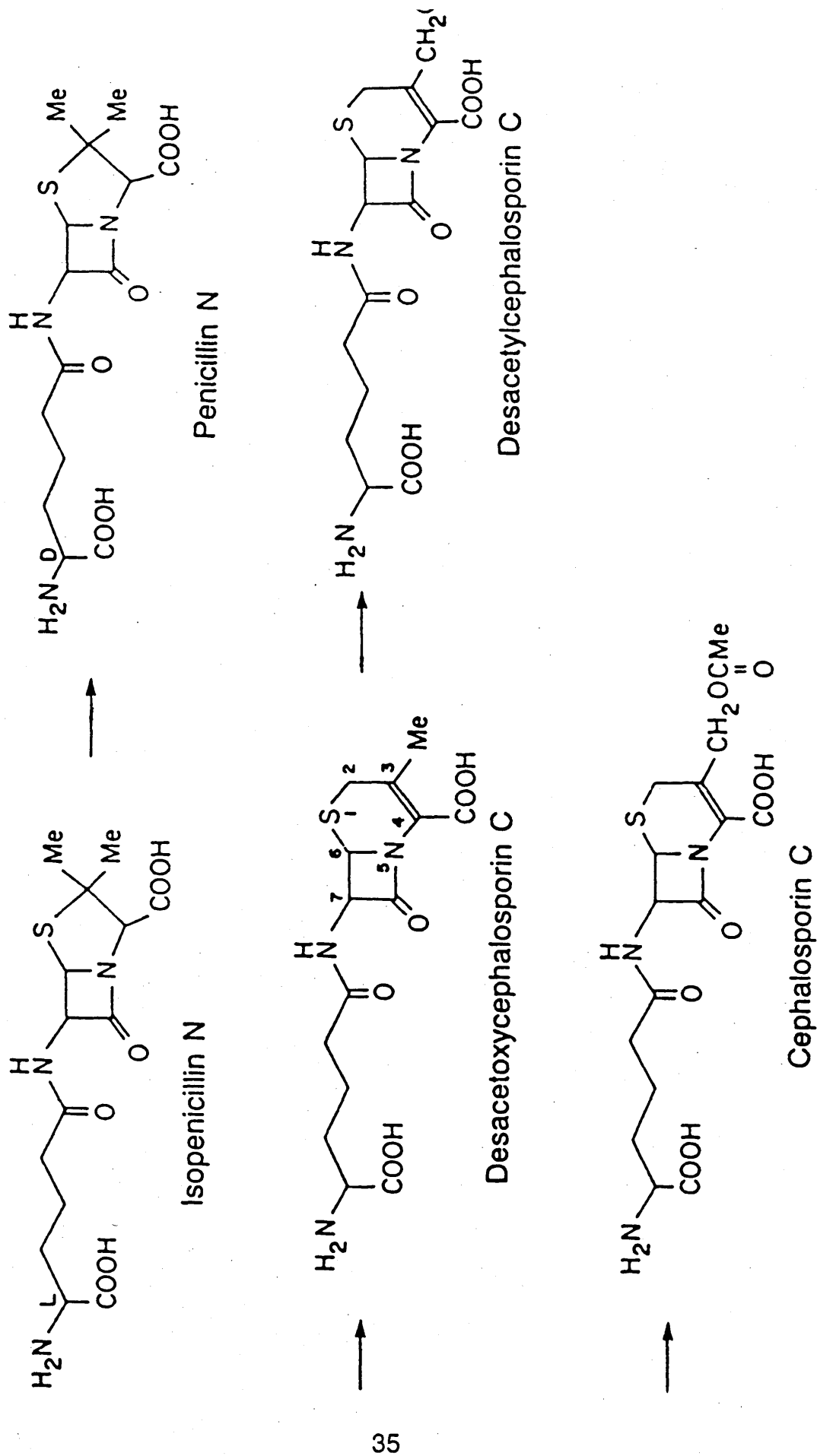


Figure 1.6 Biosynthesis of cephalosporins

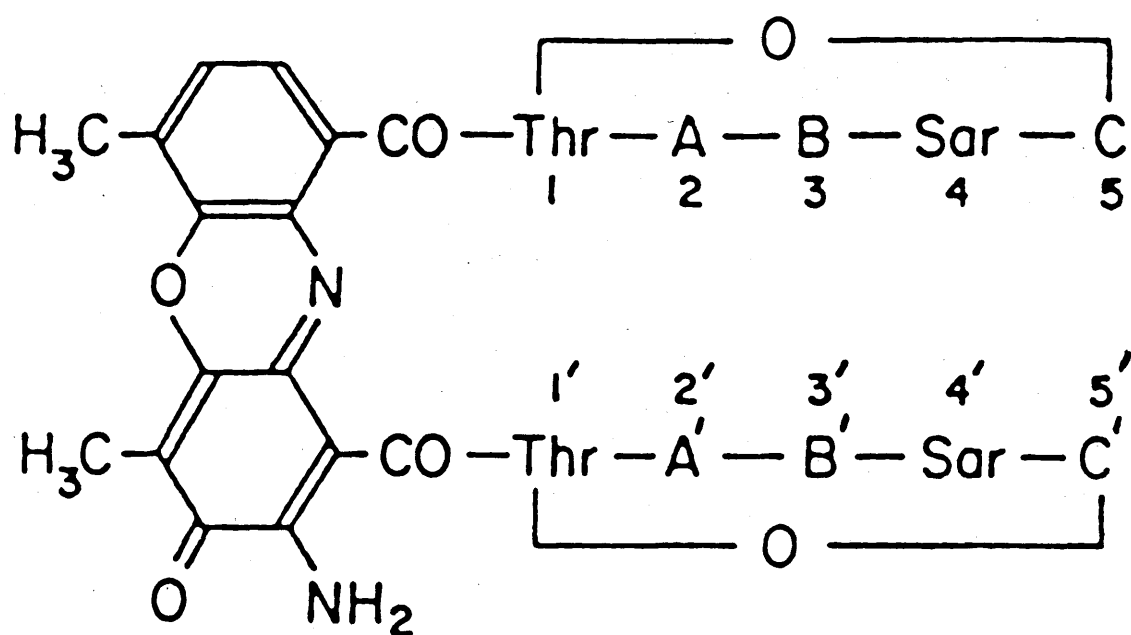


Figure 1.7 General structure of actinomycin

The sequence of the amino acids in the peptide chain differs between individual actinomycins.

1.10.2 (d) Macrolides

These antibiotics contain a 12-, 14-, or 16-membered macrocyclic lactone ring which has substituted aminosugar and/or deoxysugar groups. Erythromycin, which has a 14-membered ring structure (Figure 1.8) is a typical example.

Their main therapeutic use is against gram-negative cocci and *Mycoplasma*, with protein synthesis being their inhibitory target (they inhibit ribosomal translocation). Most macrolides currently in clinical use are produced by streptomycetes and their relatives.

The aglycone moiety of the macrolide is synthesised from acetate and propionate precursors in a manner analogous to fatty acid synthesis (Dimroth *et al.*, 1976) and macrolides constitute one type of polyketide antibiotic. Following the formation of the macrolide ring, sugars are attached to it to give the final product (Omura and Tanaka, 1983).

1.10.2 (e) Polyenes

These are a subgroup of the macrolide class of antibiotics, comprising a lactone ring of 26-38 atoms, a polyene chromophore, and usually one aminosugar group. They are produced by streptomycetes and fungi, possess potent anti-fungal activity, an example being candicidin (Figure 1.9), which is produced by *Streptomyces griseus*.

The lactone ring and aminosugar groups are synthesised in a manner analogous to that of macrolides (section 1.10.2 (d)). The polyene chromophore, containing alternating double bonds forming a ring, is usually synthesised from 4-aminobenzoic acid (PABA), derived from the shikimate pathway, via chorismate. The enzyme responsible for this conversion, PABA synthase, is a target for feedback inhibition by the aromatic amino acids (Martin,

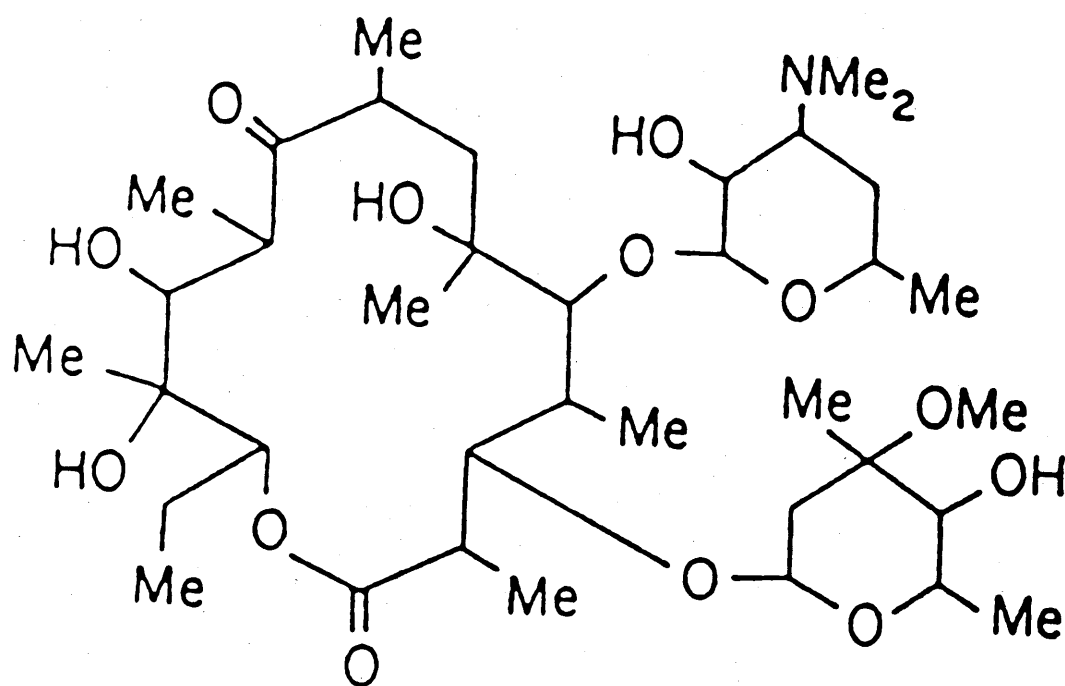


Figure 1.8 The structure of erythromycin A



Figure 1.9 The structure of candidin D

1983).

1.10.2.(f) Ansamycins

This class of antibiotic comprises an aliphatic chain, which forms an intramolecular link between two non-adjacent positions of the second component, an aromatic group. Around 20 ansamycin producers have been isolated, from either *Streptomyces* or *Nocardia* sp..

The aromatic group can either be a naphthalene or a benzene moiety, the former ansamycins having antibacterial properties (inhibiting the RNA polymerase), the latter are active against eukaryotic cells (inhibiting cell replication). The rifamycins (Figure 1.10) are the only group to have been exploited commercially.

The aliphatic chain is synthesised from acetate and propionate in a methylmalonate-malonate condensation mechanism (Dimroth *et al.*, 1976). The aromatic group precursor is derived from the shikimate pathway, although its precise identity is disputed (Lancini, 1983).

1.10.2 (g) Tetracyclines

The polyketide antibiotics are widely used in medical therapy, the most important commercial compounds being tetracycline (Figure 1.11), chlortetracycline (produced by *Streptomyces aureofaciens*) and oxytetracycline (from *Streptomyces rimosus*). They are synthesised by the condensation of acetate units to form a 19-membered chain which is then aromatised before further modification yields the final tetracyclic product.

Tetracyclines exhibit their antibacterial action by binding to ribosomes and hence inhibiting bacterial protein synthesis (Behal, 1983).

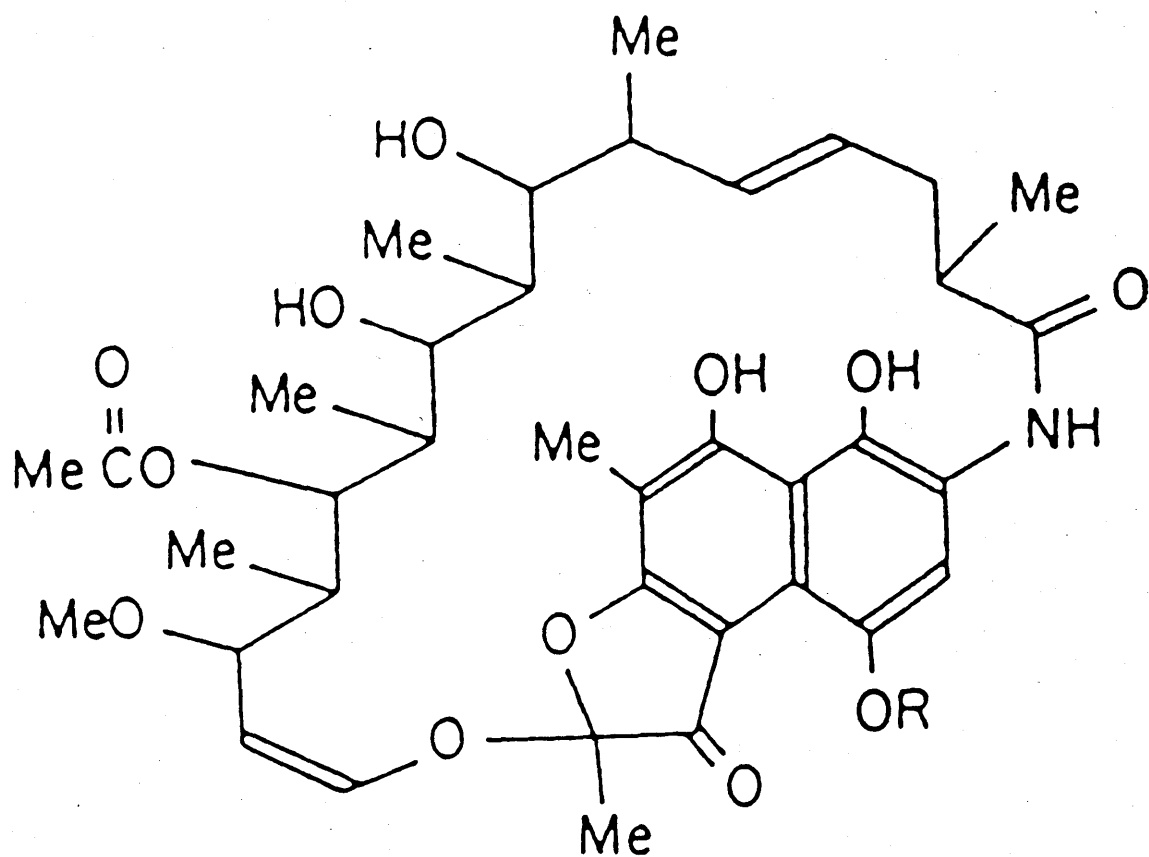
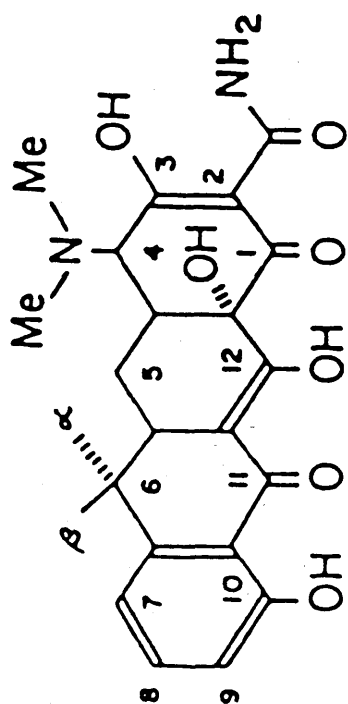


Figure 1.10 General structure of rifamycin

The functional group at the R position differs between individual rifamycins.



| | 5 | 6 α | 6 β | 7 |
|-----------------------------|----|-----------------|-----------|----------------------------------|
| Tetracycline | H | CH ₃ | OH | H |
| Chlortetracycline | H | CH ₃ | OH | Cl |
| 6-Demethyltetracycline | H | H | OH | H |
| 6-Demethylchlortetracycline | H | H | OH | Cl |
| Oxytetracycline | OH | CH ₃ | OH | H |
| 6-Deoxyoxytetracycline | OH | CH ₃ | H | H |
| Minocycline | H | H | H | N(CH ₃) ₂ |

Figure 1.11 Structures of the major tetracyclines

1.10.2.(h) Anthracyclines

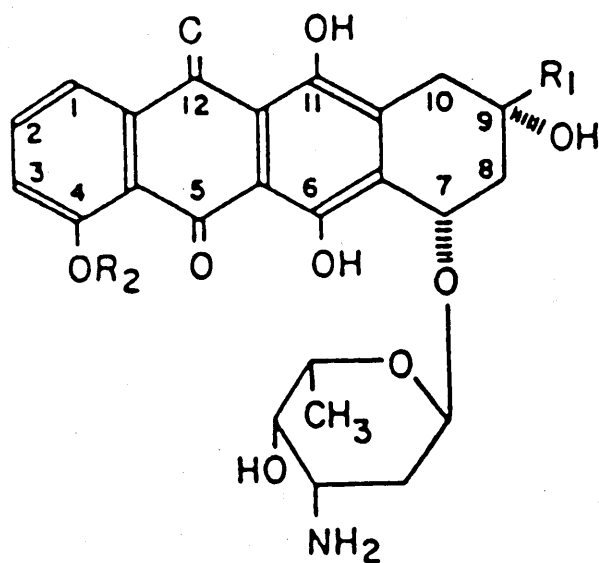
These antibiotics resemble tetracyclines in their possession of an anthraquinone chromophore, but in addition one or more sugar groups may also be attached (Figure 1.12). They are produced by streptomycetes, exhibit potent antibacterial activity, are highly toxic, and are mainly employed clinically in cancer treatment. This potent antitumour activity is a consequence of the ability of the anthracyclines to intercalate within double-stranded DNA (White, 1983).

1.10.2.(i) Chloramphenicol

The biosynthesis of chloramphenicol follows a less elaborate route than the antibiotics already described. Its structure consists of an aromatic ring with two substituted groups (Figure 1.13). It is produced by streptomycetes and closely related organisms, enjoys widespread clinical use, with its mode of action being to inhibit the procaryotic ribosomal peptidyl transferase activity. Chorismate is the branch-point intermediate for the synthesis of chloramphenicol (Malik, 1983).

1.10.2 (j) Aminoglycosides

The aminoglycosides comprise a large class of similar antibiotics which contain aminosugar groups. They are composed from a range of identical or related sugar molecules which undergo secondary modifications (eg. methylation) to yield a final product. They are synthesized by different bacterial genera including *Streptomyces*, *Micromonospora*, *Bacillus* and *Pseudomonas* (Davies and Yagisawa, 1983). As an example, kanamycin is shown (Figure 1.14). The primary effect of these drugs is on the ribosome, resulting in inhibition of protein synthesis. Clinically, they are important in the treatment of gram-



| | <u>R₁</u> | <u>R₂</u> |
|--------------|-----------------------|----------------------|
| | 13 14 | |
| Daunorubicin | —COMe | —Me |
| Adriamycin | —COCH ₂ OH | —Me |
| Carminomycin | —COMe | —H |

Figure 1.12 The structures of the anthracyclines daunorubicin, adriamycin and carminomycin

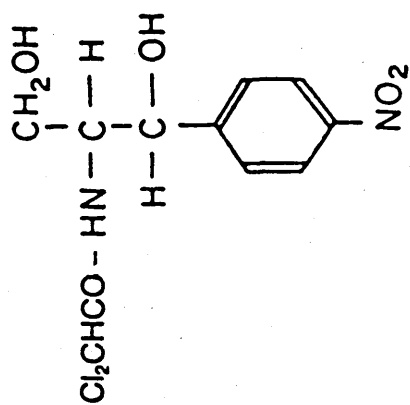


Figure 1.13 The structure of chloramphenicol

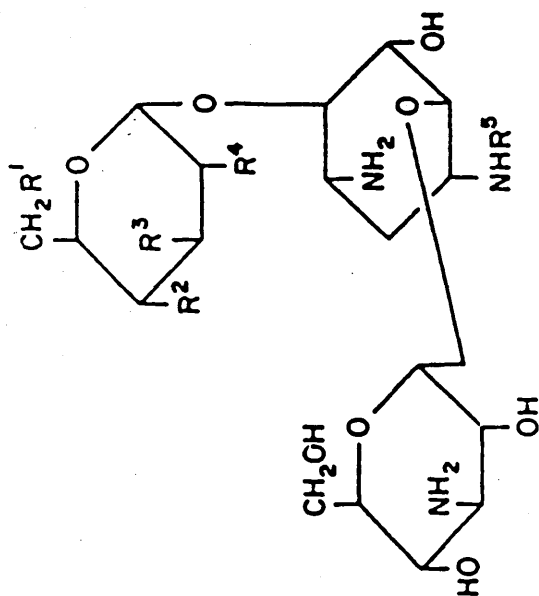


Figure 1.14 The general structure of kanamycin

The identity of R groups varies between individual kanamycins.

negative infections.

1.10.3 Possibilities for the over-production of antibiotics utilizing molecular genetics

In the past, the improvement of antibiotic yield by industrially useful microorganisms has been achieved empirically. Conventional strain improvement involves chemical or radiation mutagenesis followed by direct screening for increased yield. This approach was also employed to generate strains producing altered secondary products. In the former cases regulatory genes and in the latter case biosynthetic genes may have been altered in function.

With the advances in the understanding of the organization and regulation of antibiotic biosynthesis, and the ease with which these genes may now be isolated, a rational, planned approach to the overproduction of a single antibiotic is now possible.

The clustering of biosynthetic genes facilitates the cloning of entire pathways into streptomycete plasmid vectors using a heterologous, amenable host, such as *S.lividans*.

An increase in gene copy number and a vigorously growing host would enhance production levels.

An increased understanding of the transcriptional regulation of these gene clusters has also lead to improved productivity. There is evidence that the clusters contain pathway-specific regulatory genes, which may act positively or negatively. In the case of methylenomycin, a negative regulatory gene has been described. Disruption or deletion (Chater and Bruton, 1985 ; Fisher *et al.*, 1987) of a fragment of DNA at one end of the cluster resulted in a significant increase in production. In other cases, pathway-specific positive regulators have been identified (Ohnuki *et al.*, 1985 ; Hopwood *et al.*, 1985). Their loss correlates with a

total inability to produce antibiotic, and provision of additional copies of the regulatory gene leads to enhanced production. Interestingly, these regulatory genes can act on heterologous sets of biosynthetic genes for other polyketide antibiotics (Malpartida *et al.*, 1987).

At the next level of the hierarchy of regulation are elements implicated in morphological differentiation and antibiotic production. Pleiotrophic mutants have been identified in *S.griseus* lacking the ability to synthesize A-factor (Hara *et al.*, 1983 ; section 1.10.1), that are retarded in antibiotic production and morphological development. From mutants of *S.coelicolor* unable to synthesize several antibiotics, a DNA fragment was isolated and identified which was able to encode a putative DNA binding protein (Horinouchi *et al.*, 1986). Furthermore, extra copies of this gene stimulated antibiotic production (Horinouchi and Beppu, 1988).

As mentioned above (section 1.10.1) multiple promoters and discrete RNA polymerase sigma factors have been implicated in the control of secondary metabolite production. The substitution of strongly-expressing vegetative promoters at appropriate locations within an antibiotic cluster is another option for overproduction yet to be employed.

In conclusion then, the application of molecular biology to the study of pathway genes, pathway-specific regulatory elements, global regulatory elements, and the suitability of different promoters has led to the controllable overproduction of antibiotics.

The question pertinent to our interests is, at what point, following manipulation of these pathways does the provision of precursor molecules from intermediary metabolism become rate-limiting for the production of antibiotics ?

1.10.4 The shikimate pathway as a source of antibiotic precursors

As will have become apparent from the preceding sections, a wealth of information has been obtained regarding the intermediates, the genes, and the enzymes of various antibiotic pathways.

Three chemical structures predominate as precursors for commercially important antibiotics, however little or no basic work has been carried out on the pathways of central metabolism which furnish these structures. The paradox of the role of the shikimate pathway in particular deserves closer scrutiny. It has been shown (section 1.10.2) that a range of antibiotics including peptidolactones, polyenes, ansamycins and chloramphenicol, incorporate shikimate-derived precursors into antibiotic structures. During normal growth, the requirement for aromatic amino acids would not be regarded as a major metabolic route, considering the low abundance of such amino acids in most proteins, but following the cessation of growth and the initiation of aromatic-containing antibiotic production, an enormous metabolic load would be carried by the shikimate pathway.

How the pathway is regulated in these two strikingly different situations is of intrinsic as well as commercial interest. In particular the nature of the regulation of DAHP synthase and its role, if any, in control of flux through the pathway needs to be investigated.

1.11 Aims of the project

DAHP synthase has been shown to be tightly regulated by feedback mechanisms, both at the level of intact protein and transcriptionally. It is the single most important site of regulation for the shikimate pathway. The shikimate pathway and DAHP synthase in particular, have not been extensively studied in *S.coelicolor* before. An understanding of the regulation of the pathway would be of interest in determining factors influencing

provision of precursors for antibiotic biosynthesis. Although *S.coelicolor* itself does not produce aromatic containing antibiotics the principles learned could be applied to other organisms which do, alternatively the pathway for an aromatic antibiotic could be transferred to *S.coelicolor*.

The objectives of this work were therefore :-

- (1) To purify and characterise DAHP synthase kinetically and physically.
- (2) To investigate the possibility of isoenzyme forms.
- (3) To determine the nature, if any, of feedback inhibition on these forms.
- (4) To determine whether repression control was of importance.
- (5) To obtain sequence data from intact DAHP synthase.
- (6) To use these data to design oligonucleotide probes to facilitate the cloning of the enzyme gene by "reverse genetics".
- (7) To obtain sequence data and carry out a comparison with other DAHP synthase sequences.

Outwith the immediate scope of this project, the intact gene could then be used as a tool to manipulate flux through the shikimate pathway during growth and antibiotic production in an appropriate streptomycete strain.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and biochemicals

Ampicillin, benzamidine, Bis-trispropane buffer, blue dextran, bromophenol blue, Coomassie brilliant blue, erythrose 4-phosphate, ethidium bromide, ficoll, MES buffer, shikimic acid, spermidine and TES buffer were obtained from Sigma Chemical Co., Poole, UK.

Bactotryptone, casamino acids, yeast extract and Bactotryptone (agar) were obtained from Difco, Detroit, USA.

ATP, DTT, NAD^+ , NADP^+ , NADPH, phosphoenolpyruvate, phenylmethanesulphonyl fluoride (PMSF), N,N,N',N'-tetramethylethylenediamine (TEMED), and Tris buffer were obtained from Boehringer Mannheim, Lewes, UK.

Anthranilate, *p*-aminobenzoate, DMSO, phenylalanine, polyethylene glycol 8000, Triton X-100, tryptophan and tyrosine were obtained from BDH Chemicals, Poole, UK.

Agarose, isopropyl- β -D-thiogalactoside (IPTG), phenol (ultrapure), and 5-bromo-4-chloro-3-indoyl- β -galactoside (X-gal) were obtained from BRL, Gibco Ltd., Paisley, UK.

Acrylamide, bisacrylamide and SDS were obtained from FSA Laboratory Supplies, Loughborough, UK. Junlon PW110 was a gift from Honeywell and Stein Ltd., Wellington, UK.

Ammonium dehydroquininate was prepared following the procedure of Grewe and Haendler (1966) and was a gift from Professor J.R. Coggins. 3-Deoxy-D-*arabino*-heptulosonate 7-

phosphate (DAHP) was isolated from an *aroB*⁻ strain of *E.coli* (AB2847A) and prepared following the procedure of Lambert *et al.*, (1985), and was gift a from Professor J.R. Coggins.

[γ -³²P]- ATP was obtained from Amersham International plc., Amersham , UK.

Oligonucleotides were synthesised on an Applied Biosystems Model 280A DNA synthesiser using phosphoramidite chemistry by Dr. V. Math.

All other chemicals were of analytical reagent grade and were obtained from one of the following suppliers: BDH Ltd., Poole, UK ; Formachem Ltd., Strathaven, UK; FSA Laboratory supplies, Loughborough, UK; Koch-Light Ltd., Haverhill, UK.

2.1.2 Enzymes and proteins.

The following enzymes were obtained from Boehringer Mannheim, Lewes, UK.

- rabbit muscle aldolase (EC 4.1.2.1.3)
- bovine erythrocyte carbonic anhydrase (EC 4.2.1.1)
- bovine pancreas deoxyribonuclease 1 (EC 3.1.4.5)
- beef liver glutamate dehydrogenase (EC 3.1.4.5)
- pig muscle lactate dehydrogenase (EC 1.1.1.27)
- pig heart malate dehydrogenase (EC 1.1.1.37)
- rabbit muscle pyruvate kinase (EC 2.7.1.40)

A kit for molecular weight determination and the following enzymes and proteins were obtained from Sigma Chemical Co., Poole, UK.

Clostridium histolyticum clostripain (EC 3.4.22.8)

rabbit muscle fructose 6-phosphate kinase (EC 2.7.1.11)

chicken egg white lysozyme (EC 3.2.1.17)

bovine pancreas ribonuclease A (EC 3.1.27.5)

bovine pancreas trypsin (EC 3.4.21.4)

All restriction enzymes, T4 DNA ligase, and T4 Polynucleotide kinase were obtained from BRL, Gibco Ltd., Paisley, UK.

Dehydroquinase synthase, dehydroquinase and shikimate dehydrogenase were purified from overproducing plasmid *E.coli* strains by Mr. J. Greene as described by Mehdi *et al.*, (1987), Chaudhuri *et al.*, (1987a) and Chaudhuri *et al.*, (1987b), respectively.

2.1.3 Chromatography media

DEAE-Sephacel, Phenyl-Sepharose CL-4B and Sephadex G-50 were supplied by Pharmacia, Milton Keynes, UK. Phosphocellulose PII was obtained from Whatman Biochemicals, Maidstone, UK. Bio-Gel Hydroxylapatite was purchased from Bio-Rad Laboratories Ltd., Watford, UK.

2.1.4 Pre-packed media

Pre-packed Mono Q, phenyl-Superose, Superose 6 and Superose 12 columns were obtained from Pharmacia and utilised on a Pharmacia f.p.l.c. system. μ Bondapak C18 reverse phase columns were purchased from Waters Chromatography, Watford, UK and attached to a Beckman System Gold h.p.l.c. apparatus (Beckman Instruments Inc., High Wycombe, UK).

2.1.5 Bacterial strains, plasmids, phage and phage libraries

The bacterial strains are derivatives of *E.coli* K12. The bacterial strains used during this study are shown in Table 2.1. The plasmid pUC18 was obtained from Pharmacia. λ c857s7 DNA was obtained from BRL, Gibco Ltd., Paisley, UK. This DNA was cleaved with appropriate restriction enzymes and the mixture of fragments generated used exclusively as molecular weight size markers, and is referred to henceforth as λ -DNA.

The *Streptomyces coelicolor* genomic library was constructed using the λ GEM-11 replacement vector supplied by Promega Corporation, Madison, USA, by Mr. R.D. Taylor, Departments of Biochemistry and Genetics, University of Glasgow.

2.2 General methods

2.2.1 pH measurement

pH measurements were made with a Radiometer Model pH meter, using a combination electrode calibrated at room temperature.

2.2.2 Conductivity

Conductivity measurements were made at 4°C with a Radiometer Model CDM2e conductivity meter.

2.2.3 Protein estimation

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Table 2.1 Bacterial strains used in this study

| Bacterial strain | Relevant characteristics | Source or reference |
|--------------------------------------|--|--|
| <i>Streptomyces coelicolor</i> A(3)2 | | |
| JI3456 | SCP1 ^{NF} , SCP2 ⁻ | D.A. Hopwood (John Innes Institute) |
| <i>E.coli</i> | | |
| DS941 | <i>recF lacIq lacZdelM15</i> | Stirling <i>et al.</i> , 1988 |
| NM621 | <i>supE thy⁺ recD</i> | Whittaker <i>et al.</i> , 1988 |

2.2.4 Spectrophotometric measurement of nucleic acid

Nucleic acid concentrations were determined spectrophotometrically at 260 nm (Sambrook *et al.*, 1989). In a 1 cm path length an absorbance value of 1.0 corresponds to 50 µg/ml for double stranded DNA and 20 µg/ml for oligonucleotides.

2.3 Polyacrylamide gel electrophoresis

Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970), with a 3% stacking gel and a 10% running gel. Non-denaturing electrophoresis was carried out by the method of Davis (1964) in 7% tube gels. The ratio of acrylamide : bis-acrylamide in all PAGE experiments was 30 : 0.8 and polymerization was induced by 0.03% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. After electrophoresis gels were stained for protein by either the Coomassie or silver nitrate method (section 2.3.1).

2.3.1 Protein staining

Protein was localised on gels either by staining with Coomassie blue or with silver nitrate. The Coomassie reagent was 0.1% Coomassie brilliant blue G250 in 10% (v/v) glacial acetic acid, 50% (v/v) methanol and destain reagent was 10% acetic acid, 10% methanol. These procedures were carried out at 40°C. The silver reagent was 0.8% (w/v) AgNO₃, 0.08% (w/v) NaOH, in 200 mM-(NH₄)OH. The developer reagent was 0.02% (v/v) formaldehyde, in 0.24 mM-sodium citrate. The method was adapted from Wray *et al.*, (1981). Gels were first soaked in 50% (v/v) methanol for at least 8 hours, then incubated for 8 minutes in freshly prepared silver reagent. After washing with distilled H₂O for one hour the gel was developed with fresh developer until protein bands appeared. The reaction was then terminated by washing with distilled H₂O and the gels stored in 10% (v/v) acetic acid, 10% (v/v) methanol.

2.4 Enzyme assays

Direct or coupled spectrophotometric assays were performed at 30°C in a total volume of 1 ml (unless stated otherwise). Either a Gilford/Unicam model 252 or a Philips PU 8720 uv/vis spectrophotometer was used.

2.4.1 *S.coelicolor* DAHP synthase

(a) Stopped assay

S.coelicolor DAHP synthase was assayed by a modification of the method of Doy and Brown (1965). DAHP was treated with periodate to give β -formylpyruvate which reacted with thiobarbiturate to give a pink chromagen with an absorption maximum at 549 nm ($E_{549\text{nm}} = 6.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The assay mixture contained 50 mM-Bis(tris)propane/HCl buffer pH 7.4, 0.4 mM - phosphoenolpyruvate, 0.4 mM-erythrose 4-phosphate in a volume of 0.2 ml.

(b) Coupled continuous assay

DAHP synthase activity was measured by coupling the formation of DAHP to the oxidation of NADPH by the intervening three enzyme activities of the shikimate pathway. The assay cocktail contained 50 mM-Bis(tris)propane/HCl buffer pH 7.0, 0.5 mM-phosphoenolpyruvate, 1.0 mM-erythrose 4-phosphate, 0.1 mM-NADPH, 10 μM -NAD⁺, 0.1 units of *E.coli* dehydroquinate synthase, 0.05 units of *E.coli* dehydroquinase and 0.05 units of *E.coli* shikimate dehydrogenase. The activity of individual coupling enzymes was measured under standard conditions (section 2.4.2). The oxidation of NADPH was monitored at 340 nm ($E_{340\text{nm}} = 6.18 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

(c) Direct continuous assay

The method of Schoner and Herrmann (1976) is based on the absorbancy difference at 232 nm between phosphoenolpyruvate ($E = 2.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) and DAHP ($E = 4.15 \times 10^2 \text{ M}^{-1}\text{cm}^{-1}$). DAHP synthase activity was measured directly as phosphoenolpyruvate was depleted. The standard assay mixture contained 50 mM-Bis-trispropane/HCl buffer, pH 7.0, 0.5 mM-phosphoenolpyruvate, 1.0 mM-erythrose 4-phosphate.

2.4.2 Coupling Enzymes

(a) Dehydroquinase synthase

DHQ synthase activity was measured by coupling the release of DHQ to the 3-dehydroquinase reaction (Lambert *et al.*, 1985). The assays contained 50 mM-potassium phosphate buffer, pH 7.0, 40 μM -NAD⁺, 0.2 mM-DAHP, 0.2 mM-CoCl₂, and 0.05 units of *E.coli* 3-dehydroquinase. The formation of 3-dehydroshikimate was monitored at 234 nm ($E_{234 \text{ nm}} = 1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

(b) 3-Dehydroquinase

3-Dehydroquinase activity was assayed by the direct measurement of 3-dehydroshikimate formation at 234 nm. The assays contained 100 μM -DHQ in 50 mM-potassium phosphate buffer, pH 7.0.

(c) Shikimate dehydrogenase

Shikimate dehydrogenase was measured in the reverse direction by monitoring the reduction of NADP⁺ at 340 nm. The assay mixture comprised 100 mM-Na₂CO₃ buffer,

pH 10.6, 4 mM-shikimate, and 2 mM-NADP⁺.

2.5 Growth of *S.coelicolor*

2.5.1 Spores

(a) Preparation of pre-germinated spores

Spores were prepared and pre-germinated following the procedure given below which is adapted from Hopwood *et al.*, (1985). The inoculum used originated from a single frozen spore stock culture. Spores were grown on slopes of sporulation agar (2% (w/v) mannitol, 2% (w/v) soya bean meal flour, 1.6% (w/v) agar) and incubated for 10 days at 30°C. The spores were then harvested from the plates (5 ml distilled H₂O per slope), filtered through cotton wool and pelleted by centrifugation (Centrax, 2500 rpm, 10 minutes). The pellet was resuspended in 5 ml of 50 mM-TES buffer/KOH pH 8 and incubated for 10 minutes at 50°C. The suspension was cooled, then 5 ml of pre-germination medium (1% (w/v) Yeast extract, 1% (w/v) Casamino acids, 0.01 M-CaCl₂) and 20 µl 5 M-CaCl₂ was added and the mixture incubated at 37°C for 3 hours with agitation. The spore suspension was then harvested as before and washed twice with 5 ml distilled H₂O. The spore pellet was finally resuspended in 5 ml of 40% (v/v) glycerol and stored at -20°C. The spore count was then determined (section 2.5.1 (c)).

(b) Preparation of fresh spores

Spores were prepared and freshly used following the procedure of Hobbs *et al.*, (1989). A single slope was prepared as before (section 2.5.1 (a)) and used to inoculate several plates of sporulation agar which were incubated as before (2.5.1 (a)). Distilled water (5 ml) was added to each plate and the surface gently scraped to release the spores. Suspensions were

harvested by centrifugation and washed twice with distilled water. Before use as inocula, the spores were adjusted to a final concentration of 2×10^6 spores/ml (section 2.5.1 (c)).

(c) Spore counts

Colony forming units were determined by plating suitably diluted spore samples on mannitol/soya agar plates. Counts were made after incubation at 30°C for 5 days.

2.5.2 Cell growth

Cultures were grown in either 2 litre conical flasks containing 400 ml of medium at 30°C on an orbital shaker at 200 rpm or 10 litre flasks containing 4 litres on a magnetic stirrer. Flasks were inoculated with 2.5×10^5 spores/ml media and cells harvested 72 hours later following centrifugation (20 minutes 5,000 rpm, Beckman 6L centrifuge). Cells grown on minimal media (section 2.6.2) were resuspended in extraction buffer (100 mM-potassium phosphate pH 7, 0.4 mM-DTT, 1.2 mM-PMSF, 3.5 mM-benzamidine) and the cells lysed by French Pressing. Cells grown on YEME (section 2.6.1) were resuspended and washed twice in 1/10th culture volume distilled H₂O prior to resuspension in extraction buffer.

2.5.3. Cell breakage

Cell pellets were resuspended in ice-cold extraction buffer (1 ml buffer/4g wet weight of cells) and broken by two passages through an automatic French pressure cell at 98 mpa (14 300 psi internal pressure). The cell was pre-cooled on ice before use (cat. no. 4-3398A, American Instruments Company, Maryland, U.S.A.).

2.6 Growth media

Unless otherwise stated all media were sterilised by autoclaving at 15 psi.

2.6.1 Rich media

The rich media used for growth of microorganisms during the course of this project are listed below :-

| Medium | Composition (per litre) |
|-----------------------------|--|
| L-broth (LB) | 10 g bactotryptone 5 g yeast extract 10 g NaCl (+ 5 ml 20% (w/v) glucose) |
| L-agar | as LB, + 15 g agar |
| λ phage top agarose | as LB, + 7 g agarose |
| 2 x YT | 16 g bactotryptone 10 g yeast extract 5 g NaCl |
| YEME | 16g malt extract 10g bacto-yeast extract 5g NaCl |

| | |
|------------------|----------------------|
| Sporulation agar | 20 g mannitol |
| | 20 g soya bean flour |
| | 16 g agar |

2.6.2 Minimal media

The minimal media used for *S.coelicolor* growth was developed by Hobbs *et al.*, (1989) and contained per litre of distilled water :

| | |
|-------|--|
| 4 g | glucose |
| 4.5 g | NaNO ₃ |
| 5 g | NaCl |
| 5 g | Na ₂ SO ₄ |
| 1 g | MgSO ₄ .6H ₂ O |
| 0.5 g | CaCl ₂ |
| 0.01g | ZnSO ₄ |
| 1.2 g | Tris buffer |
| 1 g | Junlon |
| 20 g | KH ₂ PO ₄ pH 7.2 |

The pH of the mixture was adjusted to pH 7 with NaOH prior to autoclaving at 5 psi for 50 minutes. The phosphate was autoclaved separately and added prior to inoculation. To this was added 1 ml of a filter sterilized trace salts solution containing per litre :-2.04g ZnCl₂, 1.015g MnCl₂.4H₂O, 0.310g H₃BO₃, 0.425g CuCl₂.2H₂O, 0.242g Na₂MoO₄.2H₂O, 0.238g CaCl₂.6H₂O, 8.775g FeCl₃ and 0.415g NaI.

2.6.3 Selection supplements

The following supplements were added to rich media to select and identify recombinant organisms.

(a) antibiotics

Ampicillin (Amp) was used at a final concentration of 50 ng/ml. A stock solution of 25 mg/ml was filter sterilised and stored at -20°C. Hot L-agar was cooled to 55°C before ampicillin was added. L-Amp plates were stable for at least 4 weeks if stored at 4°C.

(b) chromogenic substrates

X-gal and IPTG were used at a final concentration of 20 ng/ml. A stock solution of IPTG of 20 mg/ml was filter sterilised and stored at -20°C. A stock solution of X-gal (20 mg/ml) was made up in DMSO and stored at -20°C.

2.7 Protein sequencing and composition analysis

2.7.1 Acid-washed glassware

Glassware for protein chemistry was heated for 1 hour in 6N HCl or 6N HNO₃ then left steeping overnight. It was then rinsed exhaustively with distilled water and dried in a hot oven.

2.7.2 Preparation of peptides from *S.coelicolor* DAHP synthase

(a) Digestion with clostripain

A 16 nmol sample of the *S.coelicolor* DAHP synthase was digested with clostripain following the protocol of Mitchell and Harrington (1971). Clostripain was first activated by pre-incubation in 1 mM-calcium acetate/ 2 mM-DTT overnight at 40°C. The digestion was carried out in 75 mM-sodium phosphate buffer pH 7.6, 7.5 mM-DTT at a final protein concentration of 0.5 mg/ml. The previously activated clostripain was added to give a protein/protease ratio of 50:1 (w/w). The digest proceeded for 1 hour at room temperature and for a further 4 hours at 37°C. The reaction was terminated by freezing, then the sample was lyophilized. The resulting peptides were purified by reverse-phase chromatography on a Waters μ Bondapak C18 column.

(b) Digestion with trypsin

A 10 nmol sample of *S.coelicolor* DAHP synthase was digested with trypsin in 0.5% (w/v) (NH₄)HCO₃ pH 8.5 at a final protein concentration of 1 mg/ml. Trypsin was added to give a protein/protease ratio 50:1 (w/w). The digest proceeded for 3 hours at 37°C by which time it appeared to have gone to completion. The reaction was terminated and peptides purified (section 2.7.2(a)).

2.7.3 Gas-phase sequencing

N-terminal amino acid sequencing of intact *S.coelicolor* DAHP synthase was carried out using an Applied Biosystems model 470 protein sequencer with on-line detection of amino acid thiohydantoin by a model 120A analyser as described by White *et al.*, (1990). The machine was operated by Mrs. J. Young of ICI Pharmaceuticals, Macclesfield, UK.

2.7.4 Liquid-phase sequencing

Purified peptides of *S.coelicolor* DAHP synthase and a second sample of intact protein were sequenced on an Applied Biosystems model 4774 pulsed liquid phase sequencer with on-line detection of amino acid thiohydantoin by a model 120A analyser. The instrument was operated by Drs. M. Cusack and G.B. Curry, Department of Geology, University of Glasgow.

2.7.5 Solid-phase sequencing

Sequences of clostripain peptides of *S.coelicolor* DAHP synthase were determined on a Milligen 6600 Prosequencer with on-line h.p.l.c.. Peptides were covalently attached to Sequelon-DITC membrane discs, as described previously (Chalmers *et al.* , 1991). This facility was operated by Dr. J.N. Keen, Department of Biochemistry, University of Leeds.

2.7.6 Amino acid composition analysis

Amino acid compositions were determined by Mrs. J.Young (ICI Pharmaceuticals, Macclesfield, UK) using either an Applied Biosystems Model 420 amino acid analyser or an LKB 4151 series 2 amino acid analyser. For the former, norleucine was included as an internal standard, the sample was automatically acid hydrolysed and converted to PITC amino acid derivatives which were analysed following reverse phase chromatography. For the latter, norleucine was also included as the internal standard, the sample was manually hydrolysed (6N HCl containing phenol for 24 hours at 115°C), and amino acids separated by ion exchange chromatography and detected colorimetrically after reaction with ninhydrin.

2.8 Molecular weight determinations

Standard molecular weight proteins were chosen from the list of proteins given in section 2.1.2.

2.8.1 Subunit M_r

SDS PAGE was used to estimate the subunit M_r of the purified protein. Two mixtures of proteins were used to calculate subunit M_r , either a Sigma Chemical Co. molecular weight marker kit or a home-made kit. The proteins used to produce standard curves of R_f against $\log M_r$ are listed below, those proteins contained in the Sigma kit are indicated by an asterix.

| Protein | Subunit M_r |
|---|---------------|
| rabbit muscle myosin * | 205 000 |
| <i>E.coli</i> β -galactosidase * | 116 000 |
| rabbit muscle phosphorylase b * | 97 400 |
| rabbit muscle fructose 6-phosphate kinase | 84 000 |
| bovine albumin * | 66 000 |
| bovine liver glutamate dehydrogenase | 53 000 |
| egg albumin * | 45 000 |
| rabbit muscle aldolase | 40 000 |
| bovine erythrocyte carbonic anhydrase * | 29 000 |

Values for subunit molecular weights were taken from Weber and Osborn (1969) and Mousedale *et al.*, (1987).

2.8.2 Native M_r

Gel permeation chromatography on a Superose 12 column was used to estimate the native M_r of the purified protein. This was carried out at room temperature using a Pharmacia f.p.l.c. apparatus. The column was eluted with 50 mM-potassium phosphate pH 7, 0.4 mM-DTT (flow rate = 0.3 ml/min, fraction size 0.3 ml). The eluate was monitored at 280 nm and the column calibrated with the following proteins :-

| Protein | Native M_r |
|----------------------------------|--------------|
| pig heart malate dehydrogenase | 70,000 |
| pig muscle lactate dehydrogenase | 140 000) |
| rabbit muscle aldolase | 160 000 |
| rabbit muscle pyruvate kinase | 232 000 |
| horse apoferritin | 440 000 |

2.9 Preparation of *S.coelicolor* genomic DNA

Total DNA of *S.coelicolor* JI3456 was prepared by Mrs. M. Stone and Ms. A. Wylie (Department of Genetics, University of Glasgow) essentially as described by Hopwood *et al.*, (1985).

2.10 Small scale preparation of plasmid DNA

The method of Holmes and Quigley (1981) was used for small scale plasmid preparations. 1.5 ml of an overnight culture was harvested in a microfuge and the cell pellet resuspended in 350 μ l of lysis buffer (50 mM-Tris/HCl pH 8.0, 50 mM-EDTA, 8% (w/v) sucrose, 0.5% (v/v) Triton X-100). Lysozyme was added (25 μ l of a 10 mg/ml solution) and the mixture boiled for 45 seconds followed by centrifugation in a microfuge. The pellet was

removed using a toothpick and discarded. Plasmid DNA was precipitated by addition of 1/10th volume of 3M-sodium acetate and an equal volume of isopropanol. After chilling at -20°C for 30 minutes, plasmid DNA was recovered by centrifugation (10 minutes). The plasmid DNA pellet was resuspended in 25 µl TE (10 mM-Tris/HCl pH 7.6, 2mM-EDTA). A 2 µl aliquot was sufficient for a single restriction enzyme digest.

2.11 Isolation of bacteriophage λ DNA

2.11.1 Determination of phage titre

(a) Preparation of plating bacteria

50 ml of L-broth with 0.2% (w/v) maltose, 20 mM-MgSO₄ was inoculated with a single colony of the appropriate *E.coli* strain e.g. NM621, and grown overnight on an orbital shaker. The cells were pelleted by centrifugation (MSE 18 7,000 rpm, 5 minutes, 4°C) and resuspended in 0.5 volumes of sterile, ice cold 10 mM-MgSO₄. The cell suspension was diluted if the OD 600 was greater than 2 i.e. 1.6×10^9 cells/ml. The cells were stored at 4°C and remained viable for at least 3 weeks.

(b) Plating the phage

Serial 10-fold dilutions of λ phage stock were prepared in phage buffer (10 mM-Tris HCl pH 7.5, 10 mM-MgSO₄). Bacteriophage λ infection was achieved by adding 100 µl aliquots of each dilution to 100 µl (1.5×10^8 cells) of plating bacteria suspension. The samples were incubated at 37°C for 20 minutes. 3 ml of λ phage top agarose at a temperature of 45°C was added and the mixture was poured onto plates containing λ phage bottom agar. The plates were left to stand for 5 minutes at room temperature to allow the top agarose to harden and then incubated at 37°C overnight. The plaques were counted and the titre

determined for each dilution assayed.

2.11.2 Isolation of a single plaque

λ phage were plated out at low density eg. 100-200 plaques per 10cm square plate (see section 2.11.1). A single plaque (which may have been identified by screening procedures, see section 2.21) was stabbed out of the plate into 1 ml phage buffer. 70 μ l of DMSO was added to kill any cells. After two hours incubation at room temperature the bacteriophage particles have diffused out of the agar. An average plaque yields 10^6 - 10^7 infectious bacteriophage particles, which can be stored indefinitely at 4°C in phage buffer/DMSO without loss of viability.

2.11.3 Preparation of λ phage plate lysates

Approximately 1.6×10^8 cells, of prepared bacterial suspension were infected with 10^5 plaque forming units (pfu) and plated out as described in 2.11.1. Plates were incubated overnight or until the plaques touched one another. 3 ml of phage buffer was added to the resulting lysates and plates agitated gently at room temperature for 2 hours. The phage buffer was transferred to microfuge tubes, centrifuged to remove bacterial debris and the supernatant retained. To the supernatants, 10 μ l of 1 mg/ml DNase 1 and 10 μ l of 10 mg/ml RNase A was added and this was incubated at 37°C for 1 hour. 70 μ l of DMSO per 1 ml phage buffer was added to the λ phage lysates and they were stored at 4°C. These lysates acted as phage stocks for storage or for use in the isolation of λ phage DNA.

2.11.4 DNA Preparation from λ phage lysates

An equal volume of 20% (w/v) PEG 8000, 2M-NaCl in phage buffer was added to one volume of λ phage lysate (see 2.11.3) and incubated for 1 hour at 0°C. The precipitated

phage particles were recovered by centrifugation in a microfuge (10 minutes, 40°C) and the supernatant discarded. The pellet was resuspended in 5 µl of 10% (w/v) SDS and incubated for 5 minutes at 68°C. 10 µl of 5M-NaCl was added to the mixture which was then extracted once with an equal volume of phenol:chloroform (see 2.14.1) then extracted once with chloroform alone. An equal volume of isopropanol was added to the retained aqueous phase and following incubation at -70°C for 15 minutes the λ phage DNA was recovered by centrifugation in a microfuge (15 minutes, 40°C). The DNA pellet was then washed with 70% (v/v) ethanol, allowed to air dry and dissolved in 50 µl of 1xTE buffer. This DNA was suitable for restriction analysis, Southern blotting, and sub-cloning.

2.12 Digestion of DNA with restriction enzymes

The methods used were as described in Sambrook *et al.*, (1989). Restriction digests were carried out using the BRL React buffers which were provided with each batch of enzyme. There are ten different React buffers with a range of salt concentrations, each one suitable for a range of enzymes. Analytical digests were carried out in a volume of 10 or 20 µl at 37°C. Preparative digests were carried out in larger volumes. When DNA was digested with two restriction enzymes, the endonuclease requiring the lower salt buffer was used first. After the recommended duration of digestion the salt concentration was adjusted and the second enzyme added.

2.13 Agarose gel electrophoresis

DNA was separated at room temperature on horizontal submerged agarose gels as described by Sambrook *et al.*, (1989). The Tris-borate (TBE) and Tris-acetate (TAE) buffer systems were employed. A 0.8% (w/v) agarose gel was used to accurately size restriction fragments in the range of 0.8 - 10 kb. Samples for agarose gels were prepared by addition of 0.2 volumes of 10 mM-Tris/HCl pH 7.2, 20% (w/v) ficoll, 0.5% (w/v)

bromophenol blue and 10 mg/ml ethidium bromide. Ethidium bromide (0.5 µg/ml) was added to both gel and buffer, and stained DNA bands visualised on a long wave U.V. transilluminator (U.V. Products Inc.). Restriction fragment markers of known size (*Hind* III digested λ DNA, section 2.1.5) were run alongside the unknown fragments.

2.14 Extraction and purification of DNA samples

2.14.1 Organic solvent extraction and ethanol precipitation

Protein was removed from DNA solutions by phenol/chloroform extraction. An equal volume of TE-saturated phenol/chloroform (1:1 v/v) was added to samples which were then mixed by vortexing and centrifuged in a microfuge for 5 minutes. The upper aqueous phase was removed to a fresh microfuge tube and the process repeated. Finally, traces of phenol were removed by extraction with an equal volume of chloroform in an identical manner. The final aqueous DNA pool was made 0.3 M-sodium acetate by adding 3M-sodium acetate and 2.5 volumes of ice-cold ethanol was added. The mixture was cooled at 0°C overnight, -20°C for 1 hour, or -70°C for 15 minutes and the precipitated DNA recovered by centrifugation (12,000 rpm, 4°C, 10 minutes) in a microfuge. DNA from electroelution and phage lysate preparations was further purified by chloroform/isoamylalcohol (24:1 v/v) extraction. This was carried out as for phenol/chloroform extraction.

2.14.2 Recovery of DNA from agarose gels by electroelution

The DNA band of interest was visualised by ethidium bromide staining and a hand-held long-wavelength ultraviolet lamp. The desired DNA band was excised, placed in a piece of dialysis tubing clipped at one end, 1 x TAE buffer added and the bag clipped to seal the gel slice. The gel slice was placed in a shallow layer of 1 x TAE in an electrophoresis tank. The DNA was electroeluted out of the gel and onto the inner wall of the bag by the applied

electrophoretic voltage of 50 V over 2 to 3 hours, then the polarity of the current was reversed for 1 minute to release DNA from the wall of the bag. The DNA solution was transferred from the dialysis bag to sterile Eppendorf tubes and extracted once with chloroform/isoamylalcohol (24:1 v/v) and then recovered by ethanol precipitation (see 2.14.1.1). DNA purified in this way was sufficiently pure for cloning purposes.

2.15 Ligations

The insert DNA was digested with the appropriate restriction enzymes, purified, extracted with organic solvents and ethanol precipitated. Plasmid vector pUC18 DNA was supplied linearised by digestion with the appropriate restriction enzymes and treated with bacterial alkaline phosphatase to yield DNA ready to use. This treatment prevented self-ligation of the vector and facilitated a low-background of false positive clones.

For ligations reactions where the insert size was similar to the vector size an equal concentration of foreign DNA to vector DNA was employed. For those reactions where the insert size was much greater to the vector size, at least a four-fold excess in concentration of foreign DNA to vector DNA was typical. Depending on the size of insert, ligation mixtures contained 25 ng vector and between 25 to 400 ng insert in a final volume between 5 to 10 μ l.

Vector plus insert were pre-incubated at 45°C for 5 minutes to melt any cohesive termini that had reannealed. The ligations were performed overnight in 66 mM-Tris/HCl pH 7.6, 6.6 mM-MgCl₂, 0.5 mM-ATP, 10 mM-DTT at 16°C using 0.5 Units (Weiss *et al.*, 1968) of bacteriophage T4 DNA ligase.

2.16 Transformation of *E.coli* with plasmid DNA

2.16.1 Preparation of competent cells.

A single colony of *E.coli* DS941 from a stock minimal media plate was used to inoculate 10 ml of 2 x YT which was incubated overnight at 37°C. 0.3 ml of overnight culture was used to inoculate 30 ml of 2 x YT which was grown at 37°C for around 2 hours to an OD at 600 nm of 0.4 - 0.6. The cells were cooled on ice for 10 minutes and gently harvested (Centrax, 4000 rpm, 10 minutes). When being prepared by the CaCl₂ method (Cohen *et al.*, 1972) the cells were resuspended in one half of the original culture volume of ice-cold, sterile 50 mM-CaCl₂, 10 mM-Tris/HCl pH 8 and placed in an ice bath for 15 minutes. Following centrifugation (Centrax, 4000 rpm, 10 minutes) the cells were resuspended in 1/15th original culture volume 50 mM-CaCl₂, 10 mM-Tris/HCl pH 8 and mixed in the ratio 3:1 culture to glycerol and cells dispensed into chilled microfuge tubes in 200-400 µl aliquots. These cells were stored at -70°C.

For high transformation efficiency cells, the following steps were carried out (Hanahan, 1983). Cells were prepared and harvested as above but were then resuspended in 2.5 ml of ice cold TFB (10 mM-MES/KOH pH 6.3, 100 mM-RbCl, 45 mM-MnCl₂, 10 mM-CoCl₂, 3 mM-hexaminecobaltic chloride) and incubated on ice for 15 minutes. Then, 100 µl of DMSO was added and the cells were incubated on ice for 5 minutes. Next 100 µl of 2.25 M-DTT, 40 mM-potassium acetate pH 6.0 was added and the cells further incubated on ice for 10 minutes. Finally, 100 µl of DMSO was added, then the cells were kept on ice and used on the day of preparation.

2.16.2 Transformation of competent cells

Transformations were carried out in sterile 1.5 ml microfuge tubes. Ligation mix refers to

any DNA being used in the transforming process and usually was a ligation mixture. An aliquot of ligation mix containing up to 25 ng plasmid DNA was added to 100 µl aliquots of competent cells and the mixture was incubated on ice for at least 30 minutes. The DNA/cell mix was then heat shocked at 42°C for 2 minutes. 1 ml of L-broth was added to the tubes and they were incubated without shaking at 37°C for 1 hour. The cells were then plated onto an appropriately prepared antibiotic/chromogenic containing LB plates and incubated overnight at 37°C.

2.16.3 Selection of pUC derived recombinant clones

The pUC plasmids have been constructed as cloning vectors using β -galactosidase activity as the basis of selection (Messing, 1983 ; Norrander *et al.*, 1983 ; Yanisch-Perron *et al.*, 1985). The vector carries a segment of *E.coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β -galactosidase gene (*lacZ*). Vectors of this type are used in host cells (e.g. *E.coli*, DS941) that code for the carboxy-terminal portion of β -galactosidase, therefore though neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form active enzyme. A polycloning site has been inserted, in-frame, within the coding region of the plasmid which does not affect the complementation, however insertion of additional DNA into the polycloning site generally destroys the complementation. Active β -galactosidase cleaves the chromogenic substrate X-Gal to produce a blue chromophore, when transformed cells are grown in the presence of the non-metabolizable *lac* operon inducer IPTG. However in recombinant plasmids the ability for complementation is lost, the enzyme is inactive, and consequently the colonies appear white. False positive white colonies appear at low frequency, probably due to incorrect self-ligation of the vector (Yanisch-Perron *et al.*, 1985).

2.17 DNA transfer to nylon membranes (Southern blotting)

This method is based on that of Southern (1975). After electrophoresis the agarose gel was placed in 250 ml of 0.25 M-HCl and soaked for 15 minutes. This depurination step was omitted if the DNA fragments were less than 10 kb in size. The gel was then placed in 250 ml of denaturing solution (1.5 M-NaCl, 0.5 M-NaOH) for 1 hour. The DNA was blotted onto a nylon (Hybond-N, Amersham) membrane using alkali transfer buffer (0.15 M-NaCl, 0.25 M-NaOH) overnight then the membrane was washed in 2 x SSC (SSC = 0.15 M-NaCl, 15 mM-sodium citrate pH 7.3) as described in detail in Sambrook *et al.*, (1989). The filters were then baked at 80°C for 2 hours.

2.18 ³²P labelling of DNA

2.18.1 Labelling the 5' terminus of oligonucleotides with bacteriophage T4 polynucleotide kinase

Bacteriophage T4 polynucleotide kinase can catalyze the transfer to a free hydroxyl group of the γ -phosphate group from ATP on the 5' terminus of DNA. In a total reaction volume of 10 μ l the mixture contained : 8 pmoles of purified oligonucleotide, kinase buffer (50 mM-Tris/HCl pH8, 10 mM-MgCl₂, 5 mM-DTT, 1 mM-spermidine), 8 pmoles (γ -³²P) ATP and 10 units of T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 minutes by which time it had gone to completion. Unincorporated label was removed using gel filtration (section 2.18.2).

2.18.2 Removal of unincorporated radionucleotide

Unincorporated label from end-labelling reactions was removed by gel filtration chromatography on a 20 x 1 cm Sephadex G-50 column. Sephadex G-50 was hydrated in

1 x TE and poured columns equilibrated in 1 x TE. The reaction mixture was mixed with an equal volume of Blue Dextran dye in 1 x TE and loaded directly on top of the column. The radioactivity was monitored as it passed down the column and as it approached the bottom, fractions were collected manually. Labelled oligonucleotide was co-eluted first with the Blue Dextran dye, followed by a trough then a second peak of radioactivity corresponding to unincorporated label. The fractions containing the largest number of counts were pooled.

2.19 Hybridisation of filter bound nucleic acid

The temperature of hybridisation and the salt concentration and temperature of washing solutions was dependent on the particular experiment being carried out and precise details are given in the text. Hybond-N filters were not pre-wetted, but were placed directly into a polythene bag containing prehybridisation solution (6 x SSC, 0.05% (w/v) sodium pyrophosphate, 200 µg/ml heparin, 0.05% (w/v) SDS); the volume of prehybridisation fluid was determined by filter surface area x 0.2. The bag was placed in an agitating waterbath and the filter prehybridised for at least 4 hours at 60°C. After prehybridisation the bag was opened, two thirds of the fluid was removed and labelled oligonucleotide (section 2.18.1) was added. For colony hybridisation filters, fresh hybridisation fluid was used. The bag was resealed and the hybridisation carried out for at least 90 minutes in a shaking waterbath at the appropriate temperature.

The hybridised filters were washed in large volumes of buffer appropriate in ionic strength for different experimental conditions. The temperature of this buffer varied experimentally. After washing, the filters were autoradiographed damp (under Saran wrap) and exposed to Fuji RX film using intensifying screens at -70°C. Films were developed by a Kodak X-OMAT processor.

2.20 Screening of plasmid clones by colony hybridisation

Recombinant pUC clones were screened using a modification of the method described by Nygaard and Hall (1963). Nylon filters (Hybond-N, Amersham) were placed on duplicate agar plates containing the selective antibiotic. Bacterial colonies were crossed onto a master plate (containing antibiotic) then onto the filter containing plates. The plates were inverted and grown overnight at 37°C. Alignment marks were made on the filters. The filters were removed and placed colony side up on pad of absorbent filter paper soaked in denaturation solution (1.5 M-NaCl, 0.5 M-NaOH) and left for 7 minutes. The filters were then transferred, colony side up, to a pad of filter paper soaked in neutralising solution (1.5 M-NaCl, 0.5 M-Tris/HCl pH 7.2, 1 mM-EDTA). They were left for 3 minutes then this step was repeated with a fresh pad soaked in the same solution. The filters were then washed in 2 x SSC, transferred to dry filter paper and allowed to air dry, colony side up. Finally, the filters were baked at 80°C for 2 hours. The filter was then hybridised with a nucleic acid probe (section 2.19).

2.21 Screening a phage λ library

(a) First round screening

Cells from a prepared bacterial suspension were infected (section 2.11.1) with phage from the bacteriophage λ library at a ratio of 10^4 pfu/ 10^8 cells. 2×10^3 pfu were plated onto 10 x 10 cm petri dishes using 8 ml of 0.65% (w/v) top agarose in LB. The plates were incubated overnight at 37°C. Up to six impressions could be taken from one plate onto nylon membranes, provided time was allowed for fresh phage to diffuse to the top agarose surface. Alignment marks were made on the plate and the filter was removed and treated as described for colony filters (section 2.20).

(b) Secondary screening

Desired plaque regions or single plaques were isolated as described in section 2.11.2. A lawn of bacteria (10^8 cells) were plated onto a 9 mm circular petri dish and a loopful of phage particles was streaked onto the plate. The plates were then grown up overnight at 37°C. A dozen individual plaques from this plate were picked and crossed onto a 10 x 10 cm square plate overlaid with bacteria then grown up overnight at 37°C. An impression was taken of the plate onto duplicate nylon filters which were prepared as before (section 2.21 (a)).

When identified by hybridisation and subsequent autoradiography a plug of positive phage was stabbed from the secondary plate and stored (section 2.11.2).

2.22 Computer programs for the analysis of DNA and protein sequences.

The University of Wisconsin Genetics Computer Group (UWCGC) package of programs (Devereux *et al.*, 1984) mounted on the University of Glasgow VAX Cluster (VMS3), was used for the manipulation and comparison of DNA sequences. This package contains programs for the comparison of DNA sequences with those in GenBank and EMBL (European Molecular Biology Laboratory, Heidelberg, Germany) databases which were also mounted on VMS3.

2.22.1 UWCGC programs

WordSearch :-searches for similarity between a new sequence and any group of sequences.

Segments :-aligns and displays the segments of similarity found by WordSearch.

BestFit :- Finds the best region of similarity between two sequences, and inserts gaps if necessary to obtain optimal alignment.

2.23 Removal of radioactive probe and re-use of filters

Radiative probe was removed by one of two methods listed in the Amersham booklet "Blotting and hybridisation protocols for Hybond membranes". Filters were either incubated at 45°C for 30 minutes in 0.4 M-NaOH then transferred to a neutralisation solution (0.1 x SSC ; 0.1% (w/v) SDS ; 0.2 M-Tris/HCl pH 7.5) and incubated for a further 15 minutes at 45°C. Alternatively, a boiling solution of 0.1% (w/v) SDS was poured over the filters and then the filters incubated at room temperature until the mixture cooled. The filters were then autoradiographed to check that the probe had been removed. The filter can then be pre-hybridized and hybridized with a new probe.

Chapter 3 The purification and characterisation of
the *Streptomyces coelicolor* DAHP synthase

Addendum

Prior to the development of the purification procedures, preliminary experiments were carried out in order to identify conditions of pH and buffer which were most suitable for the maintenance of *S.coelicolor* DAHP synthase activity. These experiments are described in section 3.10.2 (page 108) and the data displayed in Table 3.5.

3.1 Introduction

The purification of DAHP synthase from a wide variety of sources has been hindered by the low levels of activity found, the inconvenient and lengthy stopped assay procedure usually employed, and the instability of the enzyme in some organisms.

This chapter describes the development of both small and large scale purification procedures which yield electrophoretically homogeneous enzyme. Other purification procedures which either failed or were only partially successful are also described. Structural, kinetic and catalytic properties of the purified enzyme are also described.

3.2 Small scale purification procedure

Unless otherwise stated all manipulations following cell breakage were executed at 4°C. The buffers used in this procedure are listed (Table 3.1).

Step 1 : Extraction and centrifugation

A 70 g batch (wet weight) of *S.coelicolor* cells was suspended in 20 ml 100 mM-potassium phosphate buffer, pH 7.0, containing 1.2 mM-PMSF, 3.5 mM-benzamidine and 0.4 mM-DTT (buffer A) and broken by two passages through a French pressure cell. This material was then centrifuged at 100 000 g for 1 hour. DAHP synthase was purified from the resulting cell-free extract.

Step 2 : Salt fractionation with (NH₄)₂SO₄

The crude extract was diluted to give a protein concentration of 10 mg/ml and solid (NH₄)₂SO₄ added to give 35% saturation (209 g/l). The mixture was stirred for 20 minutes and the precipitated proteins removed by centrifugation at 28 000 g for 20 minutes.

The supernatant was adjusted to 65% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (199 g/l) and stirred for 20 minutes. The precipitated protein was collected by centrifugation at 28 000 g for 20 minutes and resuspended in 15 ml of 50 mM-potassium phosphate buffer, pH 7, 1.2 mM-PMSF, 3.5 mM-benzamidine and 0.4 mM-DTT (buffer B) and dialysed overnight against 2 litres of buffer B. The distribution of enzyme activity in $(\text{NH}_4)_2\text{SO}_4$ fractions is illustrated in Table 3.2.

Steps 3, 4 and 5 were carried out at room temperature using a Pharmacia f.p.l.c. system.

Step 3 : Anion-exchange chromatography on Mono Q

The dialysed material was loaded onto a preparative Mono Q anion-exchange column (volume 8 ml) equilibrated in buffer B. The column was washed with buffer until the A_{280} of the eluate had returned to the base-line value. Protein was eluted from the column with an 80 ml gradient of 0-0.5 M- Na_2SO_4 in buffer B, flow rate 4 ml/minute. Fractions (2 ml) were collected, assayed for DAHP synthase and those containing the highest activity were retained (Figure 3.1).

Step 4 : Hydrophobic-interaction chromatography on phenyl-Superose

Retained fractions were made 1 M- $(\text{NH}_4)_2\text{SO}_4$ by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ and applied to a phenyl-Superose hydrophobic-interaction column (volume 1 ml) equilibrated in 50 mM-potassium phosphate buffer, pH 7, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT and 0.9 M- $(\text{NH}_4)_2\text{SO}_4$ (buffer C). After the A_{280} value of the eluate had returned to the pre-loading value a 0.9 to 0 M- $(\text{NH}_4)_2\text{SO}_4$ gradient was run in a volume of 15 ml. The column was washed at 0.5 ml/min. and 0.5 ml fractions collected. After assaying for DAHP synthase activity, appropriate fractions were retained (Figure 3.2). These fractions were either dialysed against 500 ml of 215 mM-sodium phosphate buffer, pH 6.7, 0.4 mM-DTT (buffer D) or desalted into buffer D using a Pharmacia f.p.l.c. desalting column (G - 50).

Table 3.1 Buffers

The buffers used in this Chapter are listed below.

| | |
|----------|---|
| buffer A | 100 mM-potassium phosphate, pH 7.0, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT |
| buffer B | 50 mM-potassium phosphate, pH 7.0, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT |
| buffer C | as buffer B, plus 0.9 M-(NH ₄) ₂ SO ₄ |
| buffer D | 215 mM-sodium phosphate, pH 6.7, 0.4 mM-DTT |
| buffer E | 10 mM-potassium phosphate, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT |
| buffer F | 200 mM-potassium phosphate, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT |
| buffer G | 25 mM-sodium phosphate, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT |
| buffer H | 50 mM-potassium phosphate, pH 7.0, 0.4 mM-DTT, 40% (v/v) glycerol |

Step 5 : Anion-exchange chromatography on Mono Q

The partially purified material was loaded onto an analytical Mono Q anion-exchange column (volume 1 ml) equilibrated in buffer D. Following washing with buffer D, protein was eluted with a 30 ml linear gradient of 215-320 mM-sodium phosphate (Figure 3.3). The flow rate was 1 ml/min. and 0.5 ml fractions were collected. Fractions containing the peak of DAHP synthase activity were pooled and dialysed against 50 mM-potassium phosphate buffer, pH 7, 0.4 mM-DTT, 40% (v/v) glycerol (buffer H) for long term storage at -20°C.

3.3 Summary of the purification procedure

A summary of a typical purification scheme is shown in Table 3.3.

3.4 Purity of the *S.coelicolor* DAHP synthase

The *S.coelicolor* DAHP synthase was shown to be electrophoretically homogeneous under both denaturing and non-denaturing conditions. Non-denaturing PAGE of the purified enzyme showed a single band of protein upon silver staining (Figure 3.4). Both 10% and 15% SDS gels of the purified enzyme also showed a single protein species when silver stained (Figure 3.5 : Figure 3.6).

3.5 Large scale purification procedure

Unless indicated to the contrary, all procedures were carried out at 4°C after cell breakage. The buffers used in this procedure are listed (Table 3.1).

Step 1 : Extraction and centrifugation

Table 3.2 **Distribution of DAHP synthase activity in (NH₄)₂SO₄ fractions**

Enzyme was prepared as described in section 3.2 from 16g (wet weight) of *S.coelicolor* cells.

| Fraction | Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) |
|----------|-----------------|------------------------|------------------------------|
| Crude | 136.5 | 445 | 3.3 |
| 0-30% | 17.7 | 2.3 | 0.1 |
| 30-45% | 20.7 | 87.6 | 4.2 |
| 45-65% | 27.9 | 387 | 13.9 |
| 65-80% | 2.7 | 2.3 | 0.8 |

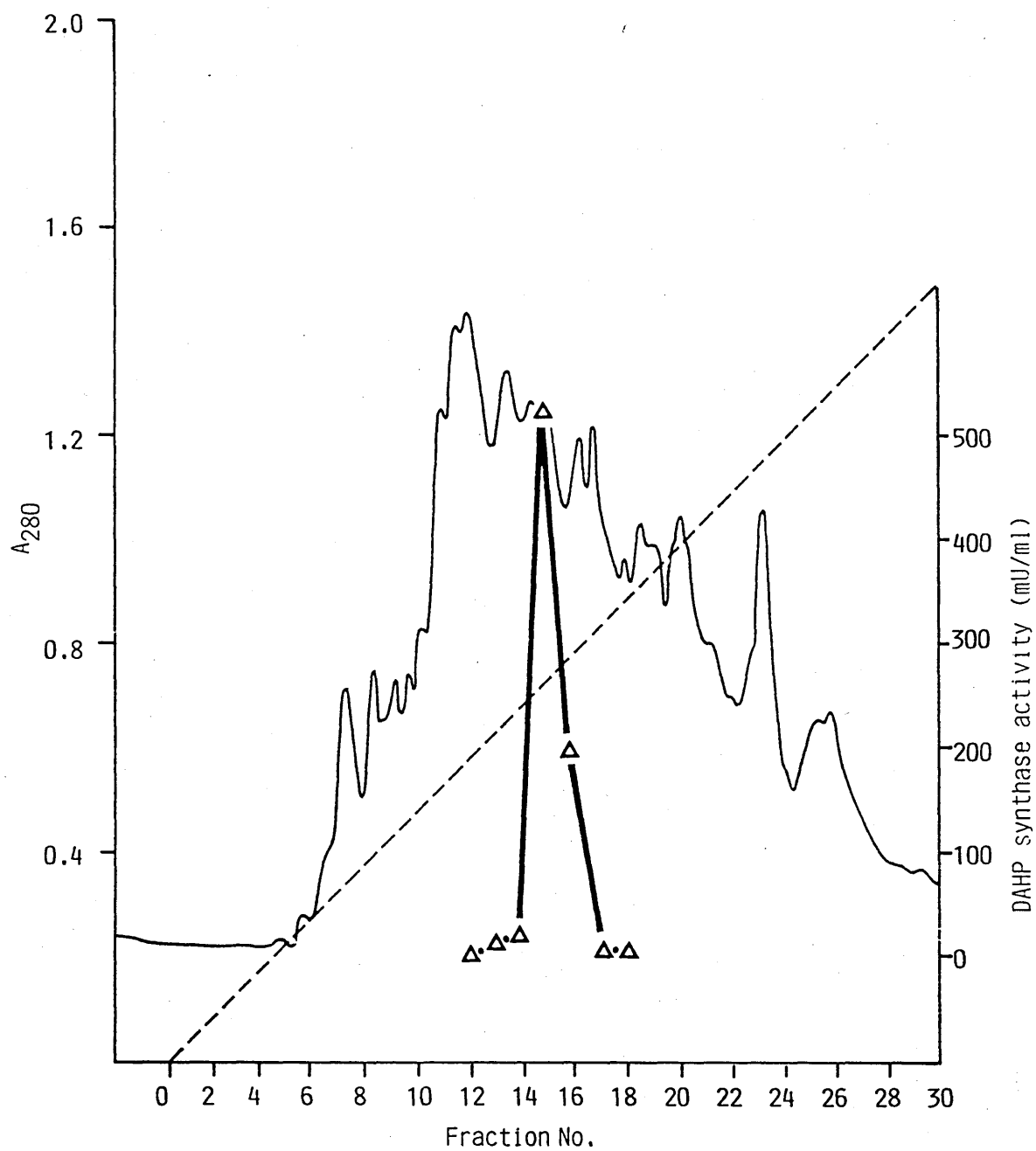


Figure 3.1 Chromatography on Mono Q

Enzyme from $(\text{NH}_4)_2\text{SO}_4$ fractionation was loaded on to a preparative Mono Q column and eluted with a sulphate gradient, as described in section 3.2.

(—) A_{280} (Δ) DAHP synthase activity (---) Na_2SO_4 gradient

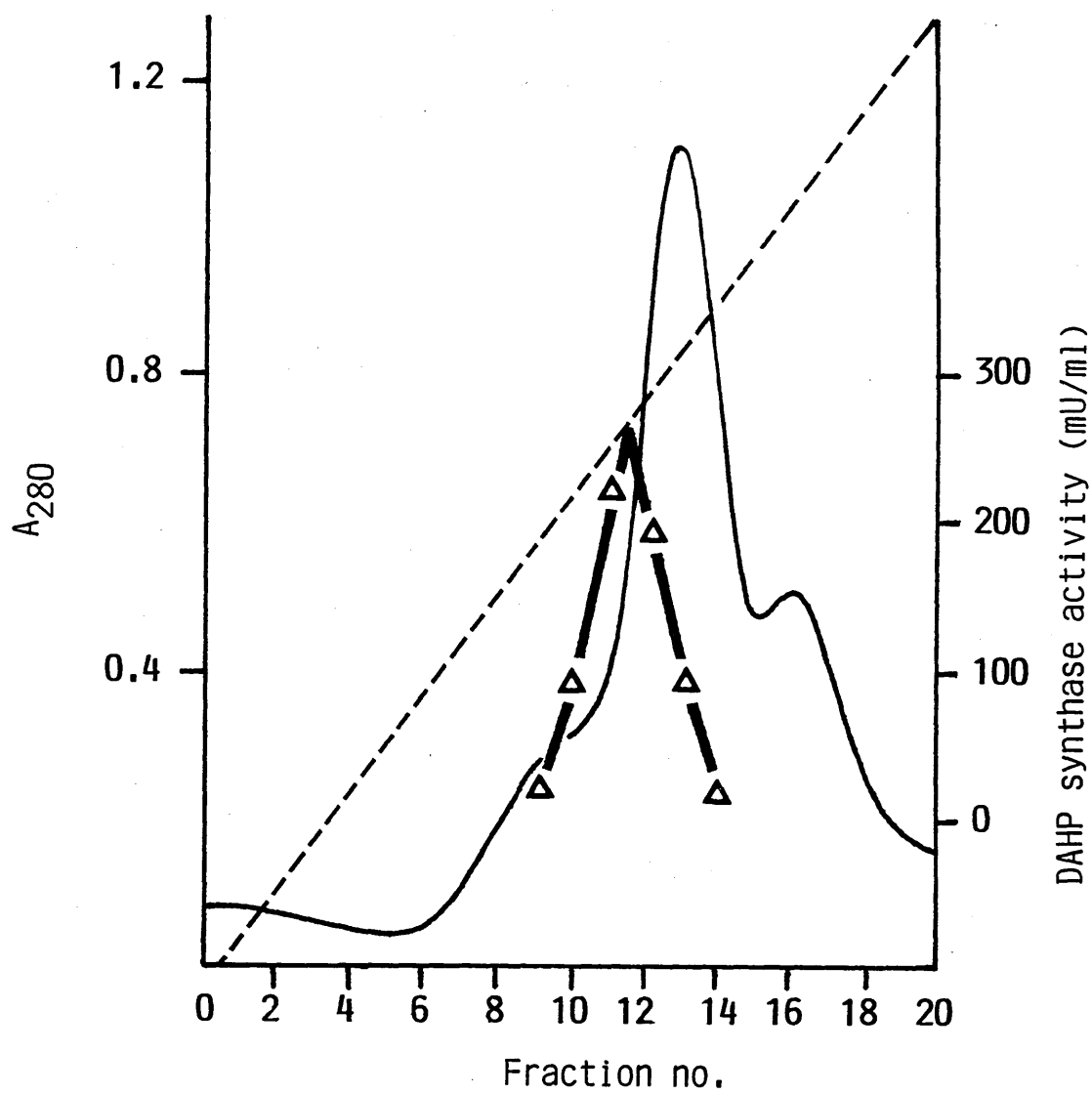


Figure 3.2 Chromatography on Phenyl-Superose

Enzyme from chromatography on Mono Q (preparative column) or chromatography on Phenyl Sepharose was loaded on to a Phenyl Superose column and eluted with a decreasing salt gradient as described in sections 3.2, 3.5.

(—) A_{280} (Δ) DAHP synthase activity (---) $(NH_4)_2SO_4$

Figure 3.3 Chromatography on Mono Q

Material from chromatography on Phenyl Superose was loaded on to an analytical Mono Q column and eluted with a phosphate gradient as described in section 3.2.

(—) A_{280} (Δ) DAHP synthase activity (---) phosphate gradient

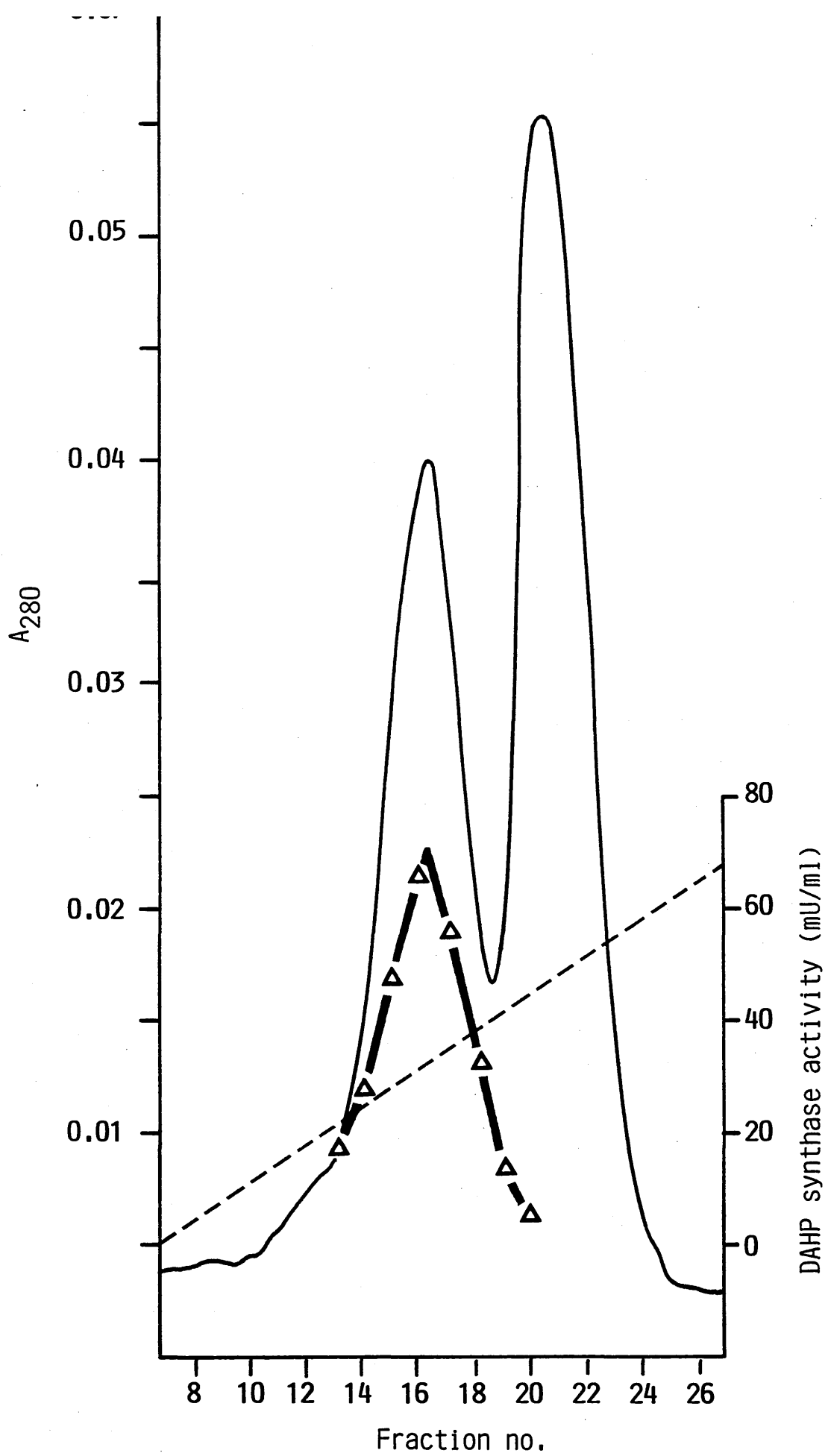


Table 3.3 Small scale purification of *S.coelicolor* DAHP synthase

The results presented are for a typical purification starting from 70g of *S.coelicolor* cells (section 3.2).

| Step | Total Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) | Yield (%) | Purification (Fold) |
|--|--------------------|---------------------|---------------------------|-----------|---------------------|
| 1. Crude | 727 | 2058 | 3.0 | 100 | 1 |
| 2. (NH ₄) ₂ SO ₄ fractionation | 224 | 1474 | 6.6 | 72 | 2 |
| 3. Mono Q chromatography | 12 | 845 | 68.4 | 40 | 25 |
| 4. Phenyl Superose chromatography | 0.4 | 532 | 1301 | 26 | 460 |
| 5. Mono Q chromatography | 0.075 | 200 | 2673 | 10 | 810 |



Figure 3.4 Native tube gel electrophoresis of *S.coelicolor* DAHP synthase

A 7% gel was run as described in section 2.3 and stained for protein by the silver nitrate method as described in section 2.3.1. Material eluted from the final chromatographic step (section 3.2) containing DAHP synthase activity was loaded onto the gel.

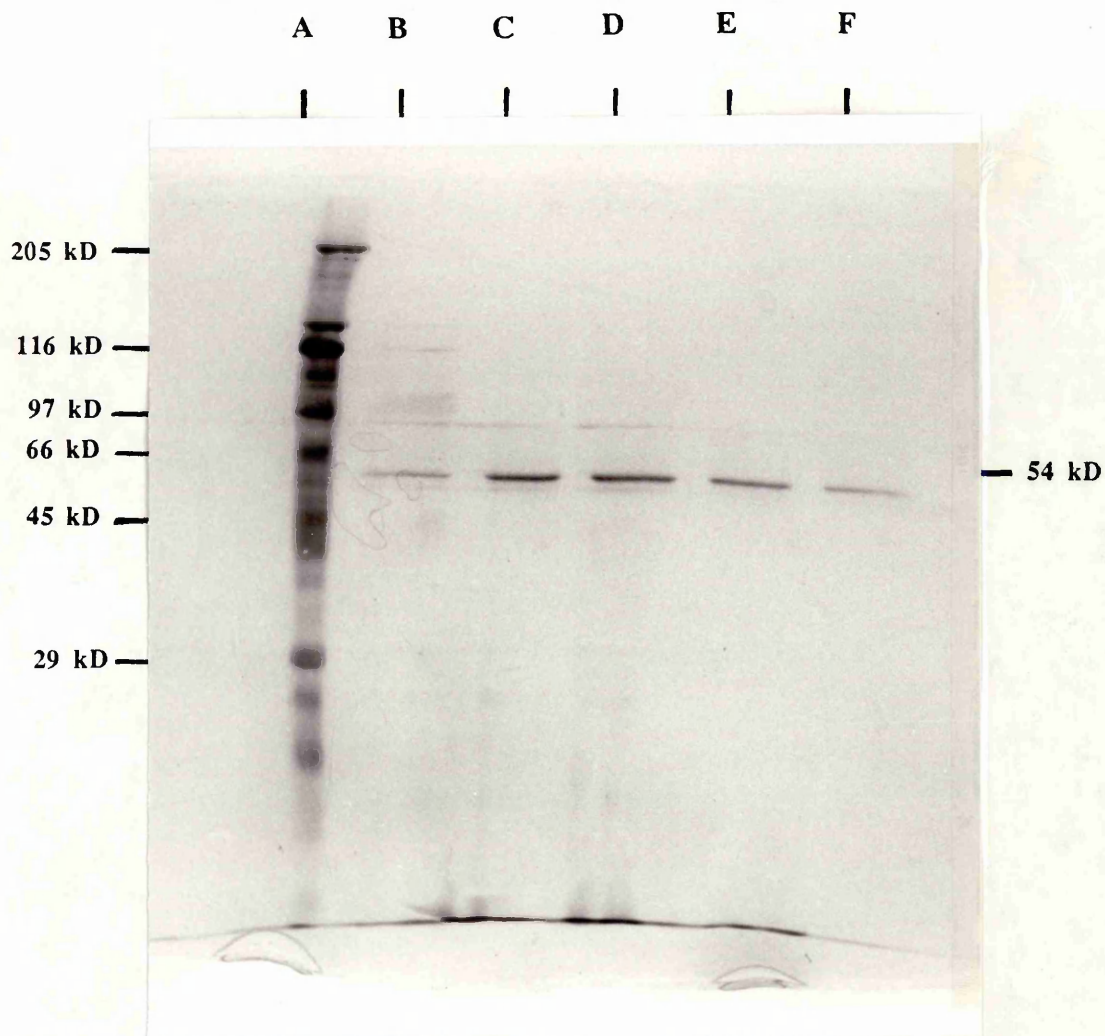


Figure 3.5 SDS PAGE of *S.coelicolor* DAHP synthase

A 10% Laemmli gel was run under denaturing conditions as described in section 2.3 and stained for protein using silver nitrate as described in section 2.3.1.

Lane A : M_r markers ; myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin, carbonic anhydrase (section 2.8.1).

Lanes B-F : material eluted from the final chromatographic column (section 3.2) containing DAHP synthase activity (Figure 3.3).



Figure 3.6 SDS PAGE of *S.coelicolor* DAHP synthase

A 15% Laemmli gel was run under denaturing conditions as described in section 2.3 and stained for protein using silver nitrate as described in section 2.3.1.

Lane A : M_r markers ; myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin, carbonic anhydrase (section 2.8.1).

Lanes B-D: material eluted from the final chromatographic column (section 3.2) containing DAHP synthase activity (Figure 3.3).

A 200 g batch (wet weight) of *S.coelicolor* cells was suspended, broken and a cell-free extract obtained as above (section 3.2).

Step 2 : Fractionation with $(\text{NH}_4)_2\text{SO}_4$

The 35-65% $(\text{NH}_4)_2\text{SO}_4$ fraction was obtained as described in section 3.2. The precipitated protein was redissolved in 15 ml buffer B and dialysed overnight against 2 x 2 litres of buffer B.

Step 3 : DEAE-Sephacel chromatography

The dialysed material was loaded onto a column of DEAE-Sephacel (bed volume 50 ml) equilibrated in buffer B. The column was washed with five column volumes of buffer B, by which time the A_{280} of the eluate was zero. The column was then eluted with a 400 ml linear gradient of 0-0.5 M- Na_2SO_4 in buffer B at a flow rate of 100 ml/hr. Fractions (10 ml) containing high DAHP synthase activity were pooled for the next step and made to 1 M in $(\text{NH}_4)_2\text{SO}_4$ (Figure 3.7).

Step 4 : Phenyl-Sepharose chromatography

The sample was loaded onto a phenyl-Sepharose column (20 ml bed volume) pre-equilibrated with buffer C. The column was washed with five column volumes of buffer C after which the A_{280} of the eluate was less than 0.1. A descending gradient of 0.9-0 M- $(\text{NH}_4)_2\text{SO}_4$ in a volume of 300 ml was run at a flow rate of 30 ml/hr and 5 ml fractions were collected. Fractions containing enzyme activity were pooled and made 1 M- $(\text{NH}_4)_2\text{SO}_4$ (Figure 3.8).

Steps 5 and 6 were carried out at room temperature using a Pharmacia f.p.l.c. system (Figure 3.2).

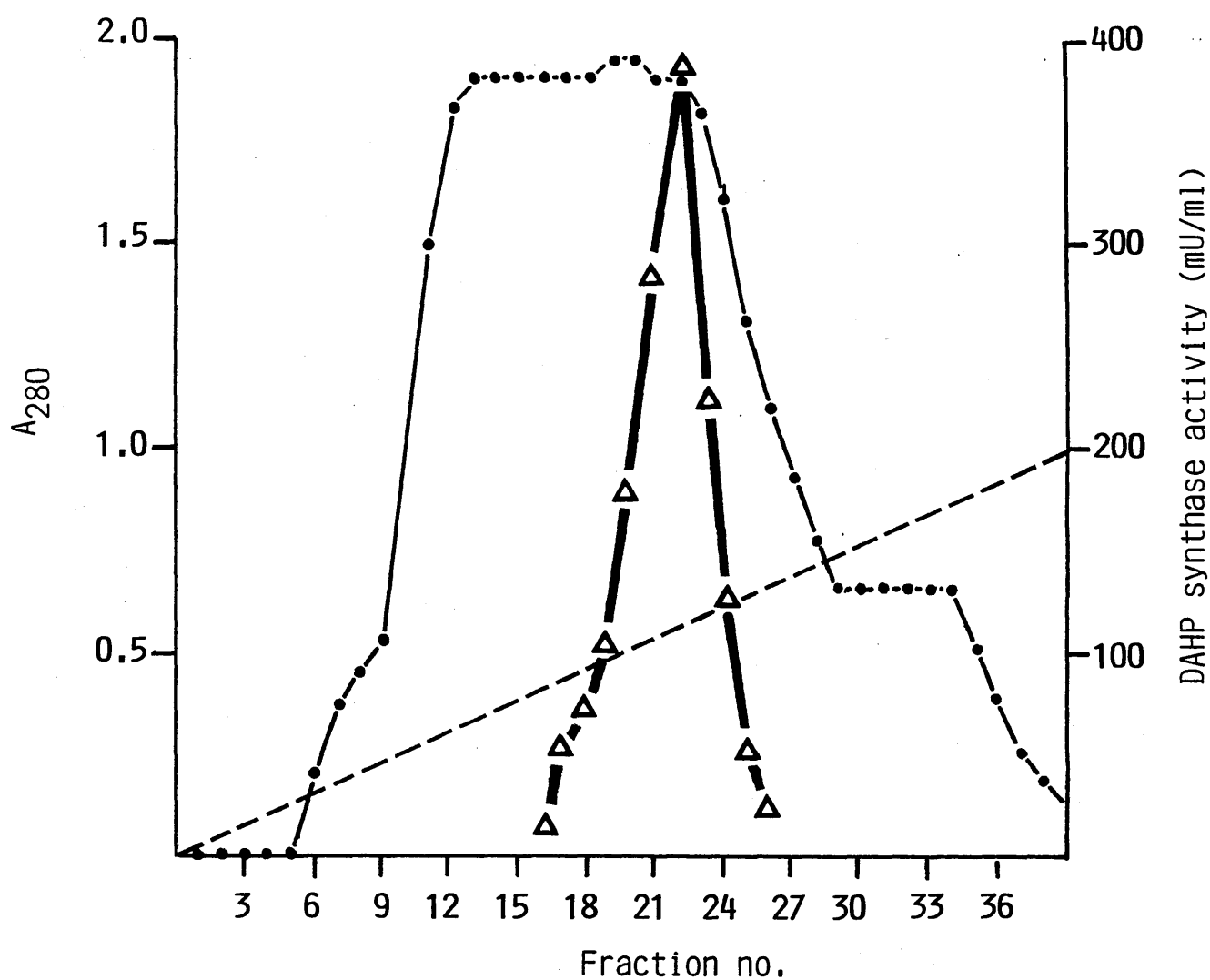


Figure 3.7 Chromatography on DEAE-Sephacel

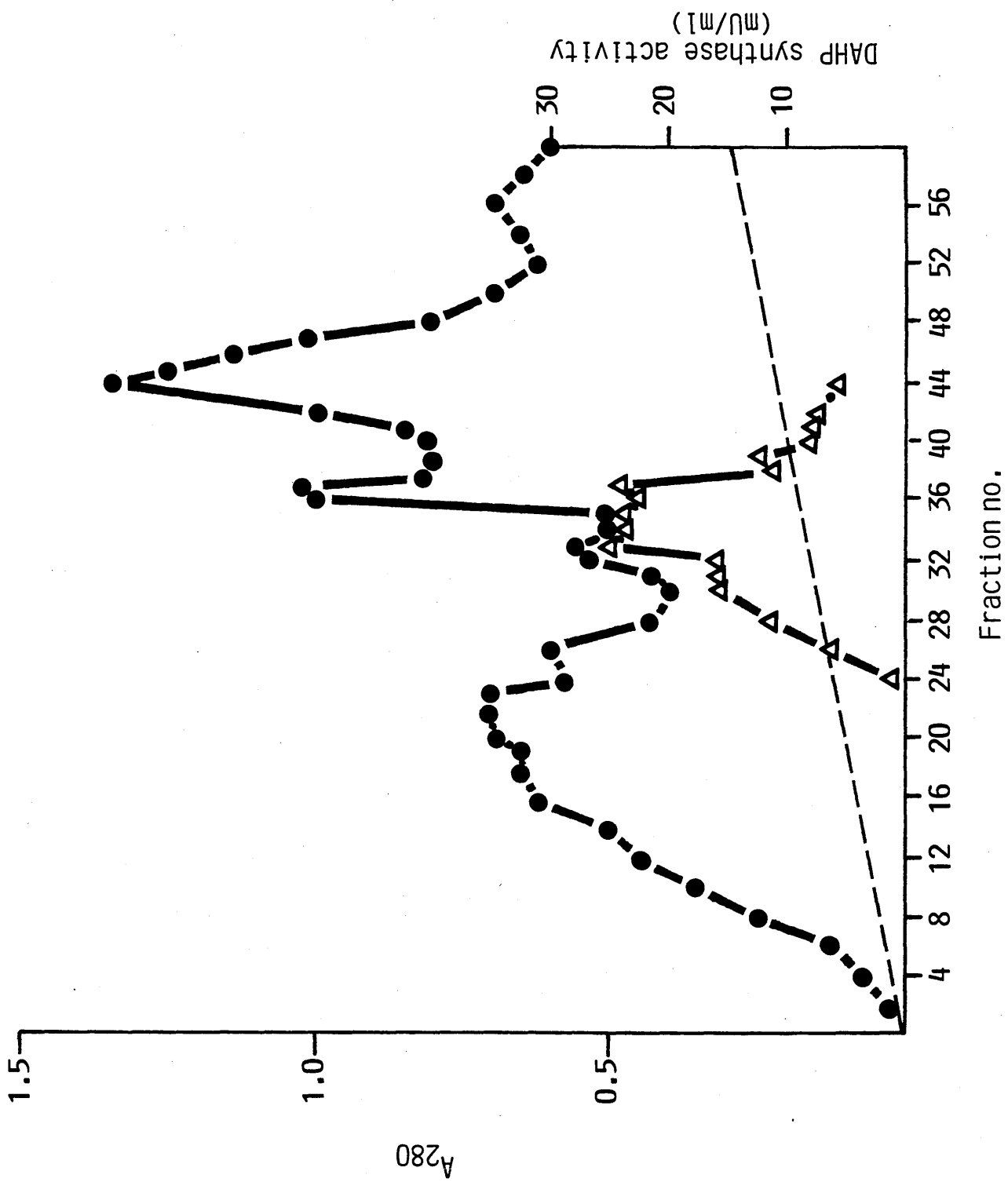
Material from the $(\text{NH}_4)_2\text{SO}_4$ fractionation was loaded onto a DEAE-Sephacel column and eluted with a sulphate gradient as described in section 3.5.

(—) A₂₈₀ (Δ) DAHP synthase activity (---) sulphate gradient

Figure 3.8 Chromatography on Phenyl-Sepharose

Enzyme from DEAE-Sepharose was loaded on to a Phenyl Sepharose column and eluted with a decreasing salt gradient as described in section 3.5.

(—) A₂₈₀ (Δ) DAHP synthase activity (---) gradient



Step 5 : Phenyl-Superose chromatography

This step was carried out as above (section 3.2).

Step 6 : Anion-exchange chromatography on Mono Q.

This was carried out as above (section 3.2) (Figure 3.9).

3.6 Summary of the purification procedure

The results obtained from a purification procedure on this scale are shown on Table 3.4.

3.7 Purity of the *S.coelicolor* DAHP synthase

The *S.coelicolor* DAHP synthase was judged to be homogeneous by reverse phase h.p.l.c. (Figure 3.10) and electrophoretic analysis (Fig. 3.11).

3.8 Other attempted purification procedures

Following the optimisation of Step 4 (section 3.2) there remained six major polypeptide species in the highest activity pool (Fig. 3.12) and prior to the development of the final purification step (section 3.2 (step 5)) several alternative chromatographic procedures were investigated. The buffers used in these procedures are listed (Table 3.1).

3.8.1 Affinity chromatography

(a) Phosphocellulose

A sample of partially purified DAHP synthase from step 4 (section 3.2) was desalted into

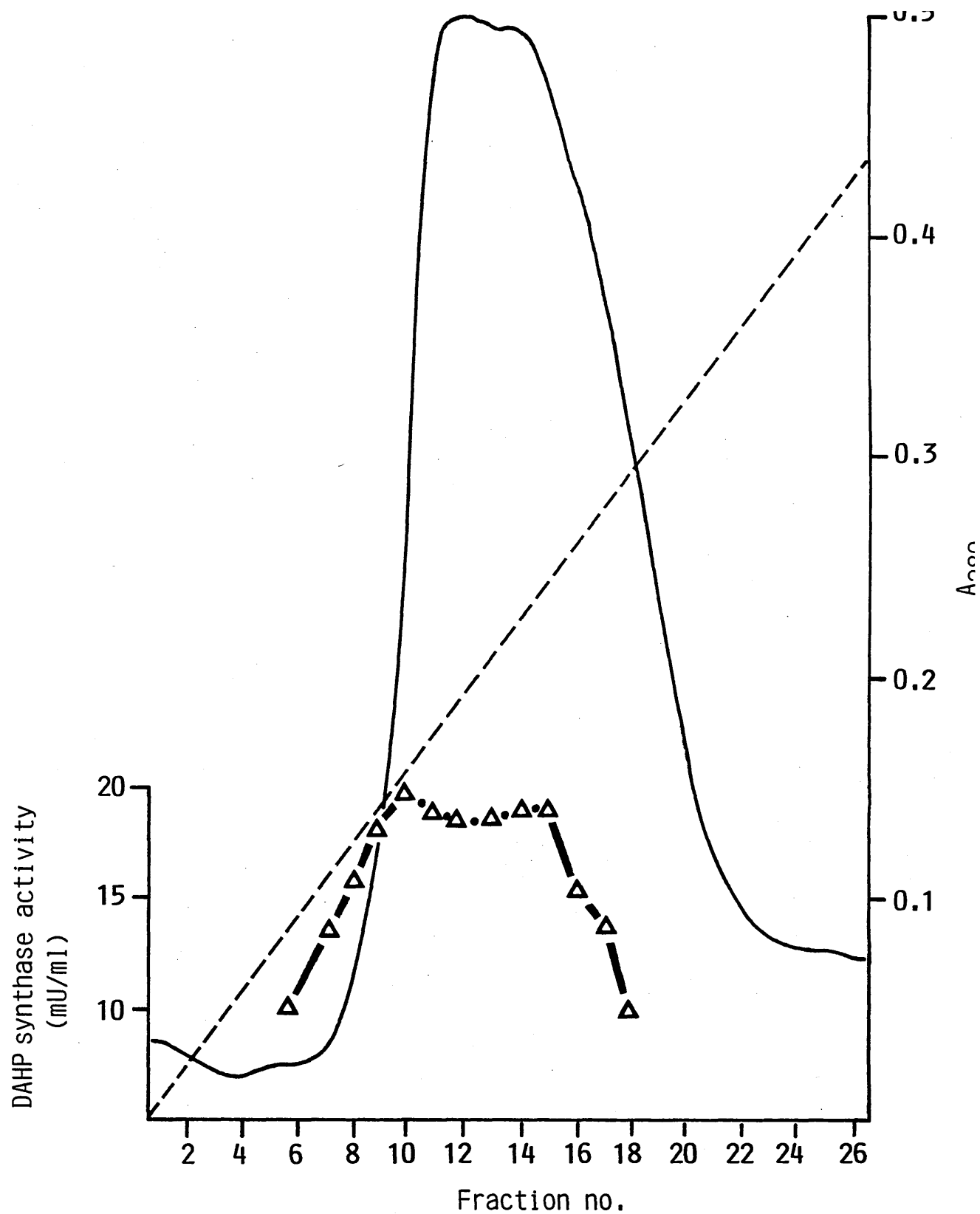


Figure 3.9 Chromatography on Mono Q

Enzyme from Phenyl-Superose was applied to an analytical Mono Q column and eluted with a phosphate gradient as described in section 3.5.

(—) A₂₈₀ (Δ) DAHP synthase activity (---) phosphate gradient

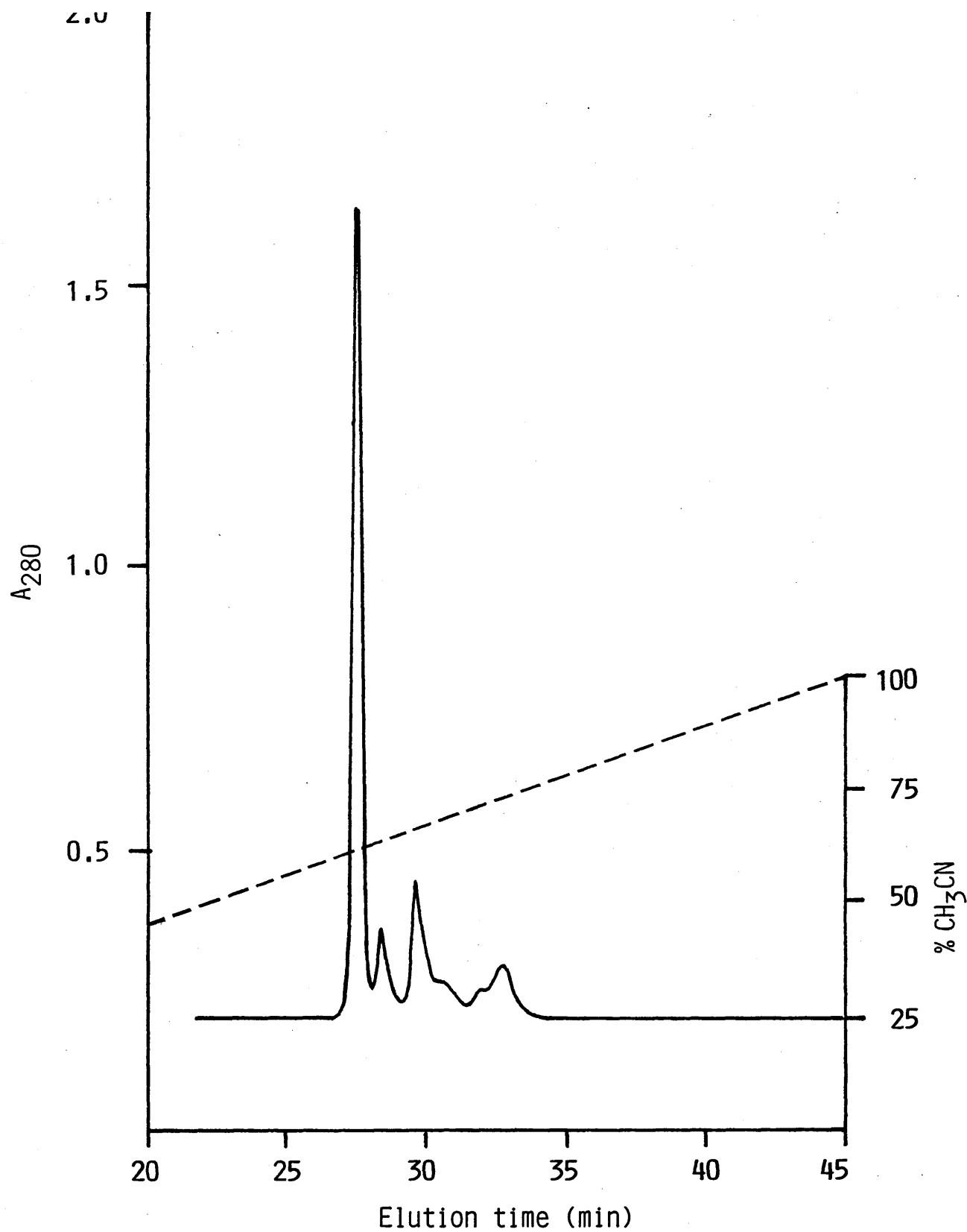


Figure 3.10 Reverse phase h.p.l.c.

Enzyme from second Mono Q pool was loaded onto a Waters C18 reverse phase column and eluted with a gradient of acetonitrile/TFA, as described in section 3.7.

(—) A₂₈₀

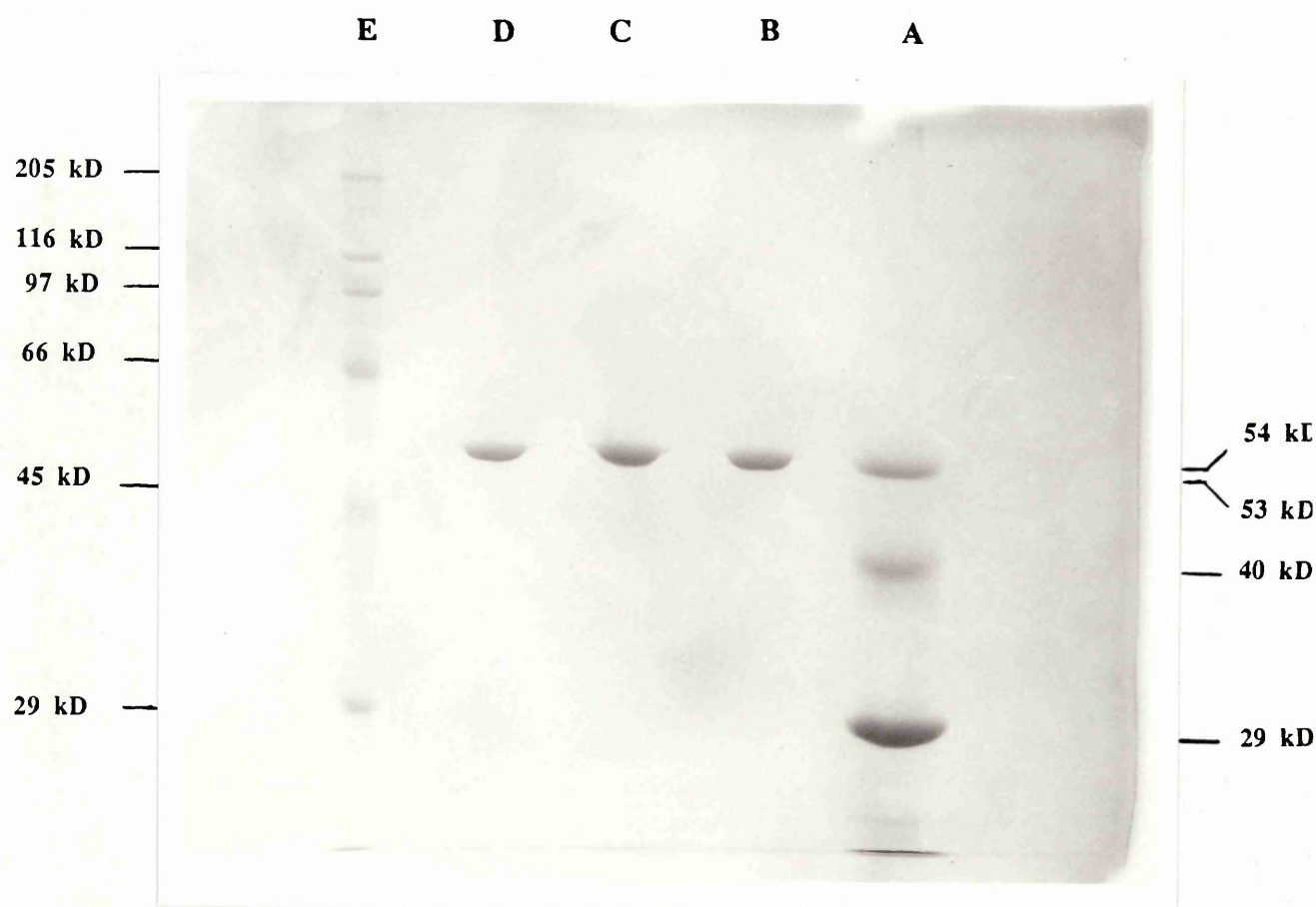


Figure 3.11 SDS PAGE of *S.coelicolor* DAHP synthase

A 10% Laemmli gel was run under denaturing conditions as described in section 2.3 and Coomassie stained for protein as described in section 2.3.1. The size of the polypeptide species is indicated.

Lane A : M_r markers ; 7 μ g each of glutamate DH, aldolase and carbonic anhydrase (section 2.8.1).

Lanes B-D : 5 μ g of material from the peak DAHP synthase activity fractions as eluted from the final chromatographic column (section 3.5) (Figure 3.3).

Lane E : M_r markers ; 2 μ g each of myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin, carbonic anhydrase (section 2.8.1).

Table 3.4 Large scale purification of *S.coelicolor* DAHP synthase

The results presented are for a typical purification starting from 200g of *S.coelicolor* cells (section 3.5).

| Step | Total Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) | Yield (%) | Purification (Fold) |
|--|--------------------|---------------------|---------------------------|-----------|---------------------|
| 1. Crude | 1415 | 6868 | 4.9 | 100 | 1 |
| 2. (NH ₄) ₂ SO ₄ fractionation | 878 | 7731 | 8.8 | 113 | 1.8 |
| 3. DEAE-Sephacel chromatography | 149 | 2551 | 17.1 | 37 | 3.5 |
| 4. Phenyl-Sepharose chromatography | 24 | 459 | 19.0 | 6.7 | 3.9 |
| 5. Phenyl-Superose chromatography | 5 | 353 | 73.5 | 5.1 | 15 |
| 6. Mono Q chromatography | 1.2 | 172 | 143.3 | 2.5 | 33 |

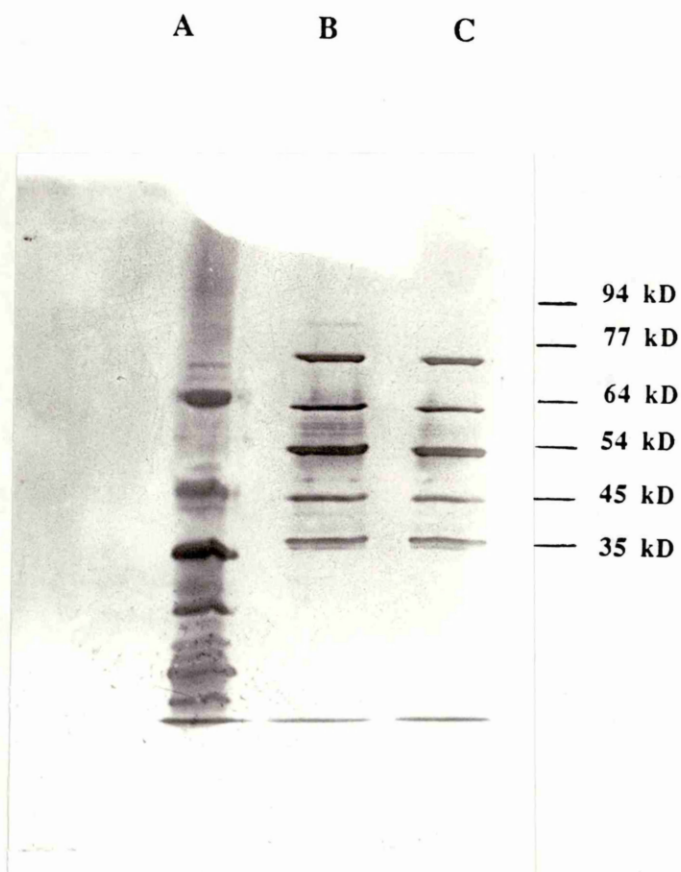


Figure 3.12 Identification of polypeptide species in the Phenyl Superose pool

A 10% SDS PAGE gel was run as described in section 2.3 and stained for protein using the silver nitrate method (section 2.3.1). The size of the polypeptide species is indicated.

Lane A : M_r markers

Lanes B,C : Peak fractions of DAHP synthase activity following the Phenyl-Superose purification step (section 3.2)

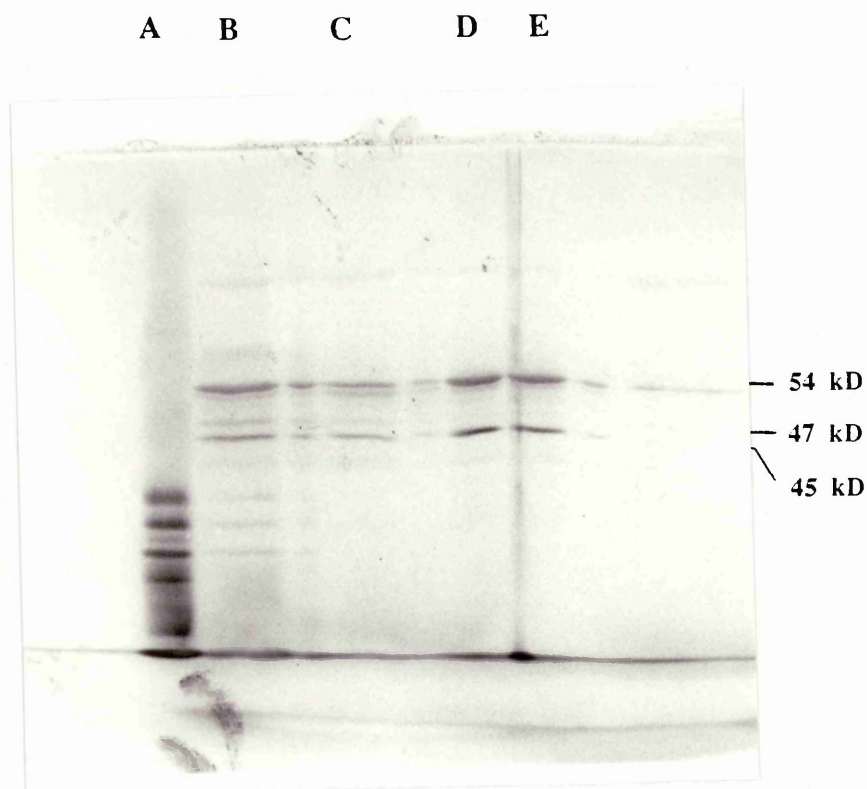


Figure 3.13 SDS PAGE analysis of hydroxylapatite and Mono S samples

A 10% SDS gel was run as described in section 2.3 and stained for protein by the silver nitrate method (section 2.3.1). The size of the identified polypeptides is indicated.

Lane A : M_r markers ; myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin, carbonic anhydrase (section 2.8.1).

Lanes B,C : peak activity hydroxylapatite fractions (section 3.8.1 (a))

Lanes D,E : flow through from Mono S column (section 3.8.3)

10 mM-potassium phosphate buffer, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT (buffer E) using an f.p.l.c. desalting column (G-50). Phosphocellulose was pre-cycled and a 5 ml volume column in a 10 ml disposable syringe was poured. The column was equilibrated with buffer E and sample loaded onto the column, which was then washed with buffer E until the A₂₈₀ of the eluate was zero. The column was then eluted with 200mM-potassium phosphate buffer, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT (buffer F) at 0.5 ml/min., collecting 2.5 ml fractions until the A₂₈₀ of the eluate was again zero. The flow through and all fractions were assayed for DAHP synthase. Under these conditions DAHP synthase failed to bind to the column and so phosphocellulose was abandoned as a potential affinity matrix.

(b) Hydroxylapatite

A 0.5 ml bed volume hydroxylapatite column was poured in a 1 ml plastic Eppendorf pipette tip and equilibrated in buffer F. Partially purified samples of DAHP synthase from step 4 (section 3.2) were desalted into buffer F as before and loaded onto the column which was then washed with a further five column volumes of buffer F. Under these conditions DAHP synthase bound to hydroxylapatite. If the molarity of buffer F was increased to 25 mM-potassium phosphate, pH 6.5, no activity was bound to the column. Substrate elution was attempted using buffer F containing a final concentration of 10 mM-PEP but this failed to remove DAHP synthase activity from the column; the activity was eluted using 50 mM-potassium phosphate buffer, pH 6.5, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT. Peak fractions contained three major polypeptide species (Figure 3.13 tracks B,C). Although some purification was achieved, substrate elution with PEP had failed to yield homogeneous enzyme and this method was not utilized further

3.8.2 Gel filtration

A Pharmacia f.p.l.c. Superose 6 gel filtration column was equilibrated in 50 mM-potassium

phosphate buffer, pH 7, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT (buffer B). The two highest activity fractions from step 4 (section 3.2) were pooled and concentrated using an Amicon Centricon 10 microconcentrator to a volume of 100 μ l and loaded onto the column. The column was washed at a flow rate of 0.5 ml/min. and 0.25 ml fractions were collected and assayed for DAHP synthase activity. The profile of eluted protein with DAHP synthase activity superimposed is shown (Figure 3.14). SDS PAGE analysis was carried out on the fractions containing enzyme activity (Figure 3.15) and the following conclusions drawn :- gel filtration chromatography had failed as a worthwhile purification step to follow step 4 (section 3.2) ; it conclusively showed that two of the polypeptide species present in peak fractions from step 4 (section 3.2) correlated with enzyme activity; the calculated subunit M_r 's as judged by SDS PAGE of the two polypeptides were 54 kDa and 52 kDa, respectively.

3.8.3 Mono S cation-exchange chromatography

Pooled hydroxylapatite fractions (section 3.3.1(b)) were desalted into 25 mM-sodium phosphate buffer, pH 6.7, 0.4 mM-DTT, 3.5 mM-benzamidine and 1.2 mM-PMSF (buffer G) and loaded onto a Pharmacia analytical Mono S cation-exchange column equilibrated with buffer G. The column had been previously washed with buffer G containing 0.5 M-NaCl. The column was washed with buffer G and eluate collected and assayed. The eluate contained DAHP synthase activity, and after SDS PAGE analysis (Fig. 3.13, tracks D,E) was shown to have the same polypeptide composition as the loaded material. Under these conditions DAHP synthase had failed to bind to the column, and no other contaminating polypeptides had bound to the column, consequently this purification method was not pursued further.

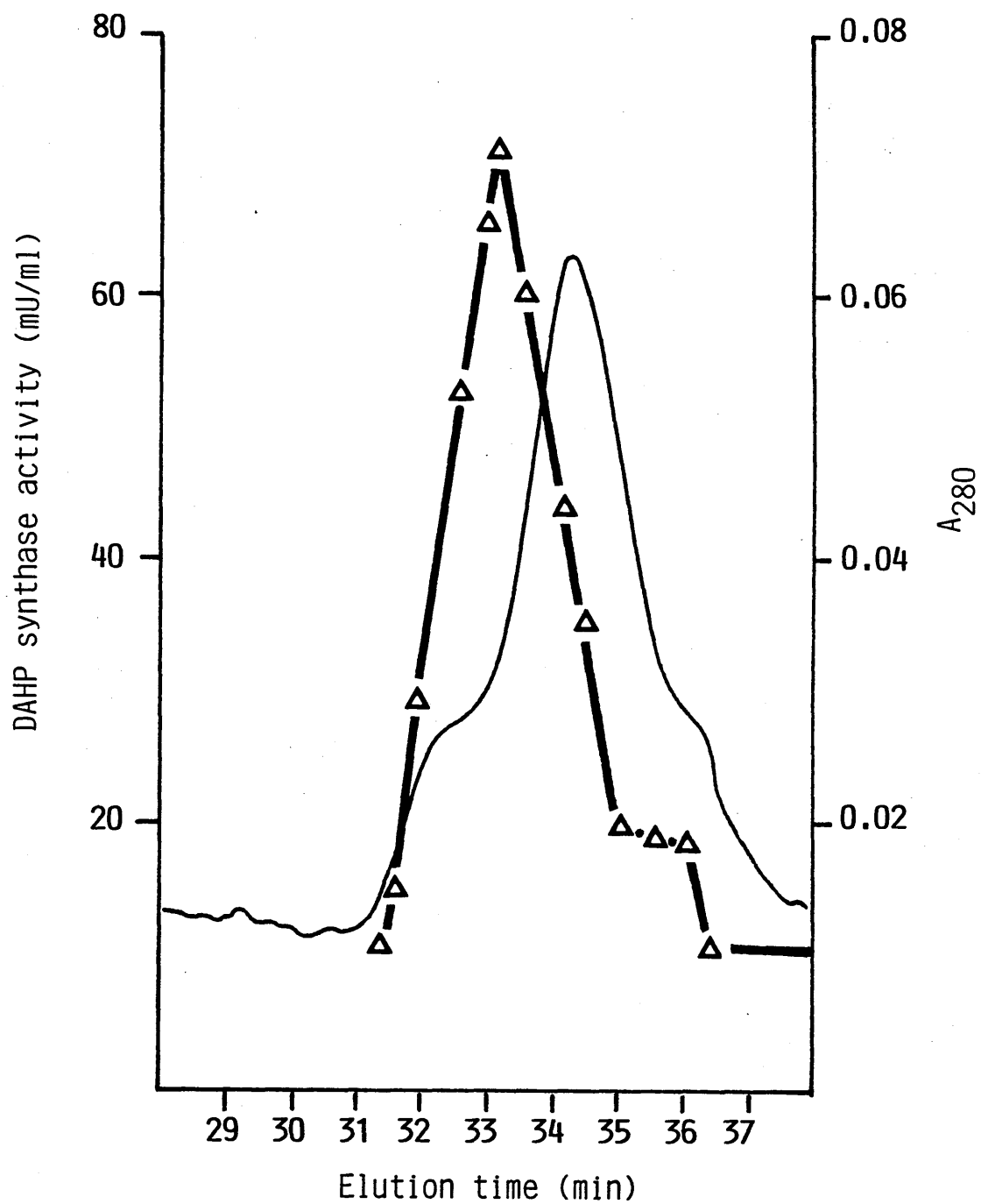


Figure 3.14 Chromatography on Superose 6

Material from Phenyl-Superose (section 3.2) was applied to a Superose 6 gel permeation chromatography column as described in section 3.8.2.

(—) A₂₈₀ (Δ) DAHP synthase

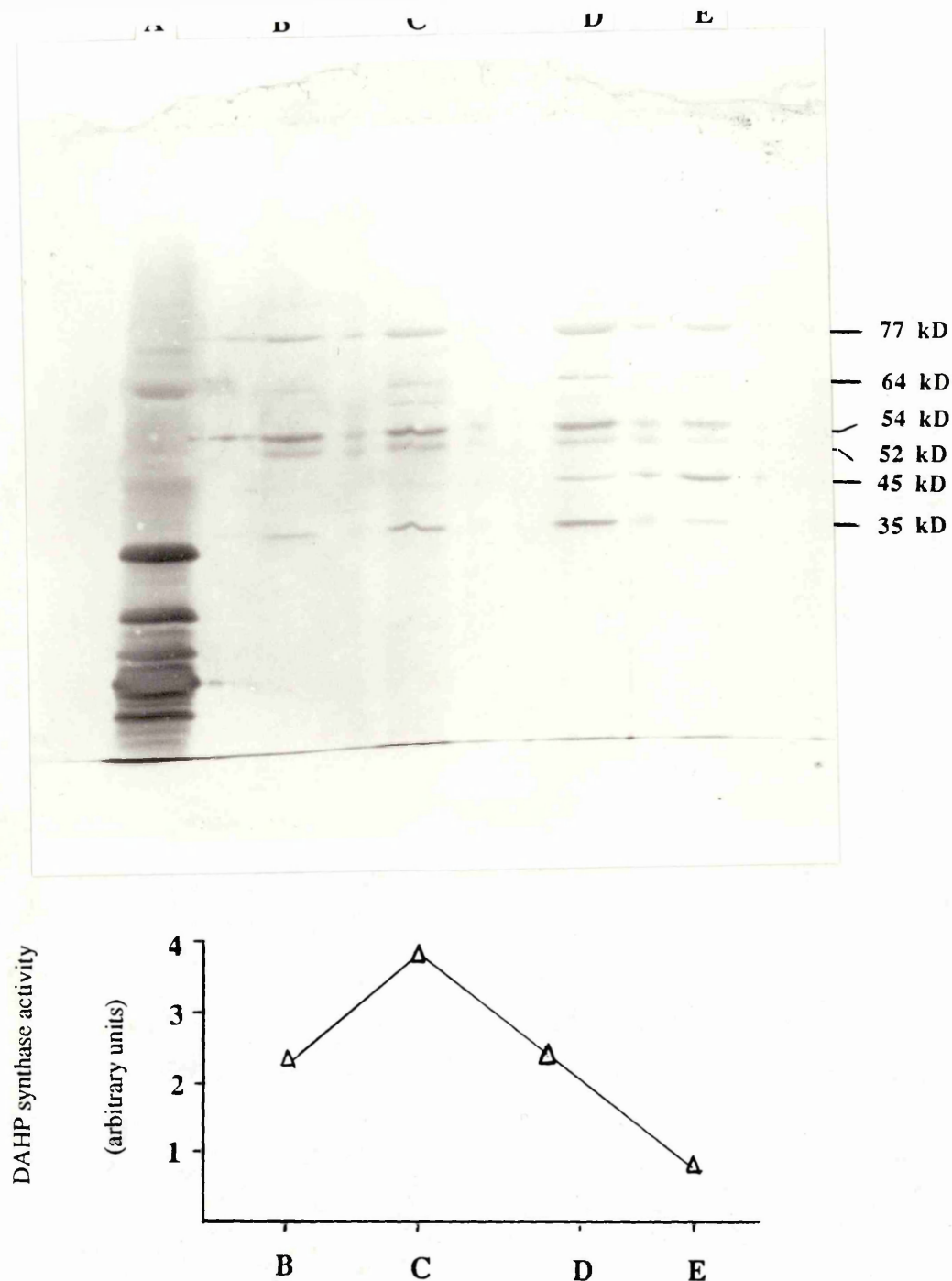


Figure 3.15 SDS PAGE analysis of Superoose 6 fractions

A 10% SDS gel was run as described in section 2.3 and stained for protein by the silver nitrate method (section 2.3.1). The size of the identified polypeptides is indicated.

Lane A : M_r markers ; myosin, β -galactosidase, phosphorylase b, BSA, ovalbumin, carbonic anhydrase (section 2.8.1).

Lanes B-E : samples from across a protein peak with corresponding DAHP synthase activity.

3.9 Molecular weight determination

Molecular weight determinations of the *S.coelicolor* DAHP synthase were undertaken in order to determine the quaternary structure of the enzyme. Gel filtration experiments utilizing high performance chromatography were used to determine the native M_r . The subunit M_r of *S.coelicolor* DAHP synthase was determined by SDS PAGE.

3.9.1 Subunit M_r of the *S.coelicolor* DAHP synthase

The subunit M_r of the purified enzyme was determined under denaturing conditions by comparing electrophoretic mobility with the mobility of standard proteins of known M_r (Figure 3.16). Comparison of the mobility of DAHP synthase with these standards gave a subunit M_r of 54 000.

3.9.2 Native M_r of the *S.coelicolor* DAHP synthase

Standard proteins of known M_r were used to calibrate a Superose 12 gel filtration column which was run on a Pharmacia f.p.l.c. system. A standard curve of peak V_e against molecular weight was constructed (Figure 3.17). Purified fractions from step 3 (section 3.2) were loaded onto the column in a final volume of 200 μ l, the column was washed with buffer B at 0.3 ml/min. and 0.3 ml fractions collected and assayed. The measured peak V_e for DAHP synthase corresponded to a M_r of 107 000 indicating that the native enzyme is a dimer.

3.10 Kinetic properties of *S.coelicolor* DAHP synthase

Kinetic analysis of *S.coelicolor* DAHP synthase was carried out using either assay method (b) or (c) (section 2.4.1) and unless stated otherwise the concentrations of substrates were at a saturating level (section 2.4.1).

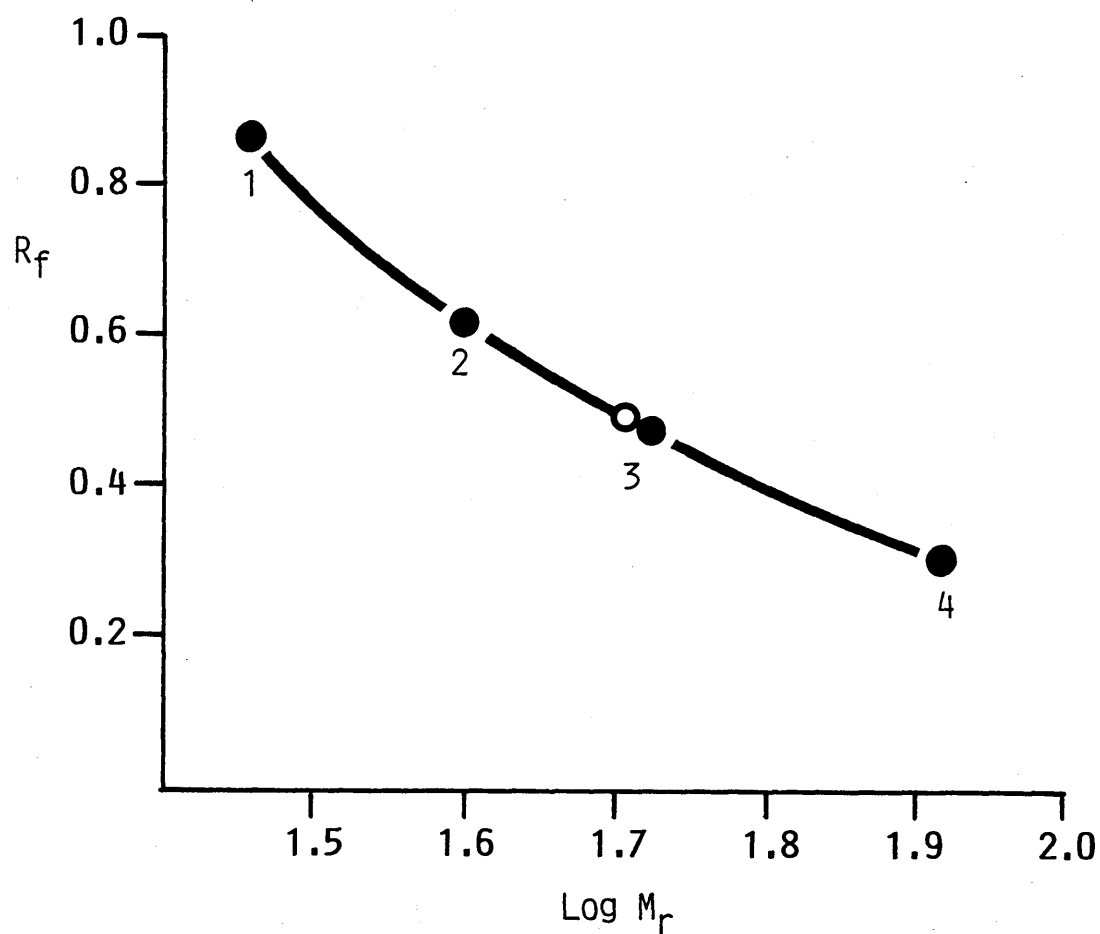


Figure 3.16 SDS PAGE standard curve

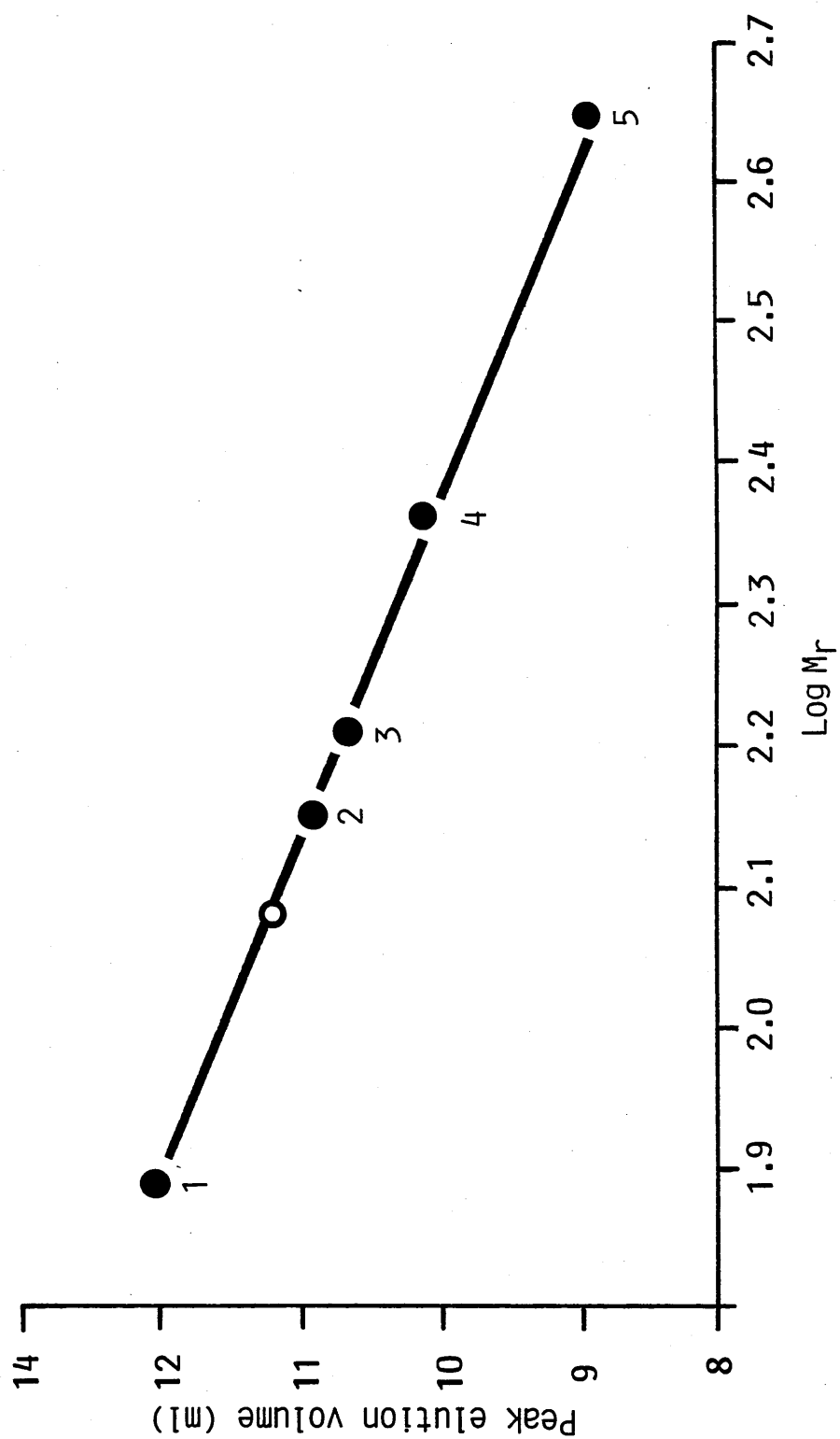
SDS PAGE (10%) was performed as described in section 2.3. The R_f values of standard proteins (section 2.8.1) were calculated and plotted against the $\log M_r$. The R_f of the purified *S.coelicolor* DAHP synthase is shown (o). The standard curve was fitted by eye.

| | |
|---------------------------------|--------------|
| (1) carbonic anhydrase | 29 000 M_r |
| (2) aldolase | 40 000 M_r |
| (3) glutamate DH | 53 000 M_r |
| (4) fructose 6-phosphate kinase | 84 000 M_r |

Figure 3.17 Superose 12 gel filtration standard curve

A Superose 12 gel filtration column was calibrated and run as described in section 2.8.2. Peak elution volume (ml) was plotted against $\log M_r$. The standard curve was fitted by eye.

| | |
|----------------------------|---------------|
| (1) malate DH | 70 000 M_r |
| (2) lactate DH | 140 000 M_r |
| (3) aldolase | 160 000 M_r |
| (4) pyruvate kinase | 232 000 M_r |
| (5) apoferritin | 440 000 M_r |
| (o) DAHP synthase activity | |



3.10.1 Substrate kinetics

The initial velocity (V) was determined as a function of the concentration of one substrate at a fixed saturating level of the other substrate. First PEP concentrations were kept constant at a saturating level of 0.5 mM and initial velocities of the reaction were measured with varying amounts of E4P (Figure 3.18). Then, comparable data were obtained with varied PEP concentrations and a fixed E4P concentration of 1 mM (Figure 3.19). Sets of data were determined using both continuous assay systems and an apparent K_m for E4P of 280 μ M and an apparent K_m for PEP of 100 μ M derived. K_m values were greater when the coupled assay method (section 2.4.1(b)) was used with a phosphate containing buffer, with inorganic phosphate exhibiting product inhibition. PEP acted as a normal Michaelis-Menten substrate, showing hyperbolic saturation (Figure 3.20) whereas E4P acted as an inhibitor at concentrations above 1.1 mM (Figure 3.21), this inhibition being competitive with respect to PEP (Figure 3.22).

3.10.2 The effect of pH on the reaction velocity and enzyme stability

The influence of pH upon enzyme activity and stability is shown. Partially purified enzyme was assayed directly under saturating conditions at different pH intervals in bistrispropane/HCl buffer. Activity increases rapidly with increasing pH to an optimum between pH 7 to pH 7.4 ; further increases in pH lead to a strong decrease in activity (Figure 3.23).

(NH₄)₂SO₄ pellet aliquots were dialysed overnight against 50 mM-potassium phosphate buffer, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT at various pH intervals with activity found to be most stable at pH 7 (Table 3.5). Enzyme assays and enzyme purification procedures were consequently maintained around pH 7 wherever possible.

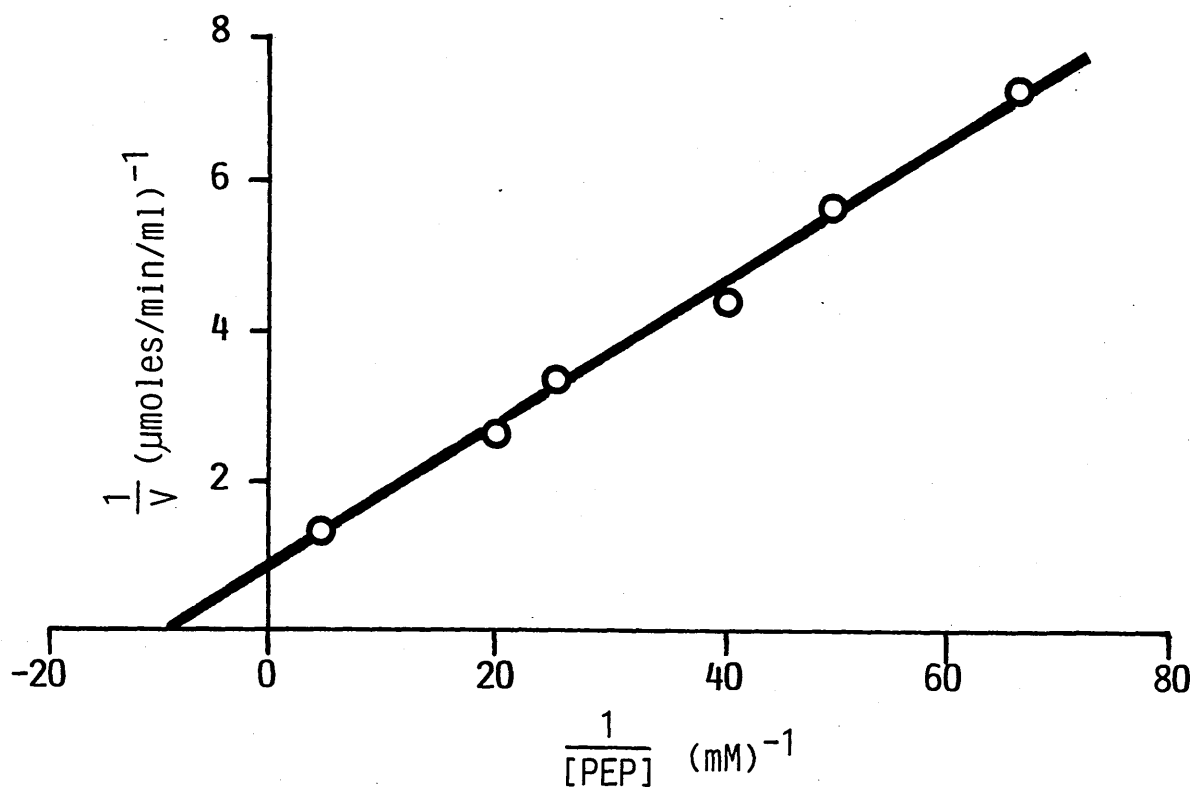


Figure 3.18 Determination of apparent K_m of DAHP synthase for PEP

Double-reciprocal plot of initial velocity against PEP concentrations at 1mM-E4P. DAHP synthase was assayed either by the direct or the coupled continuous method as described in section 3.10.

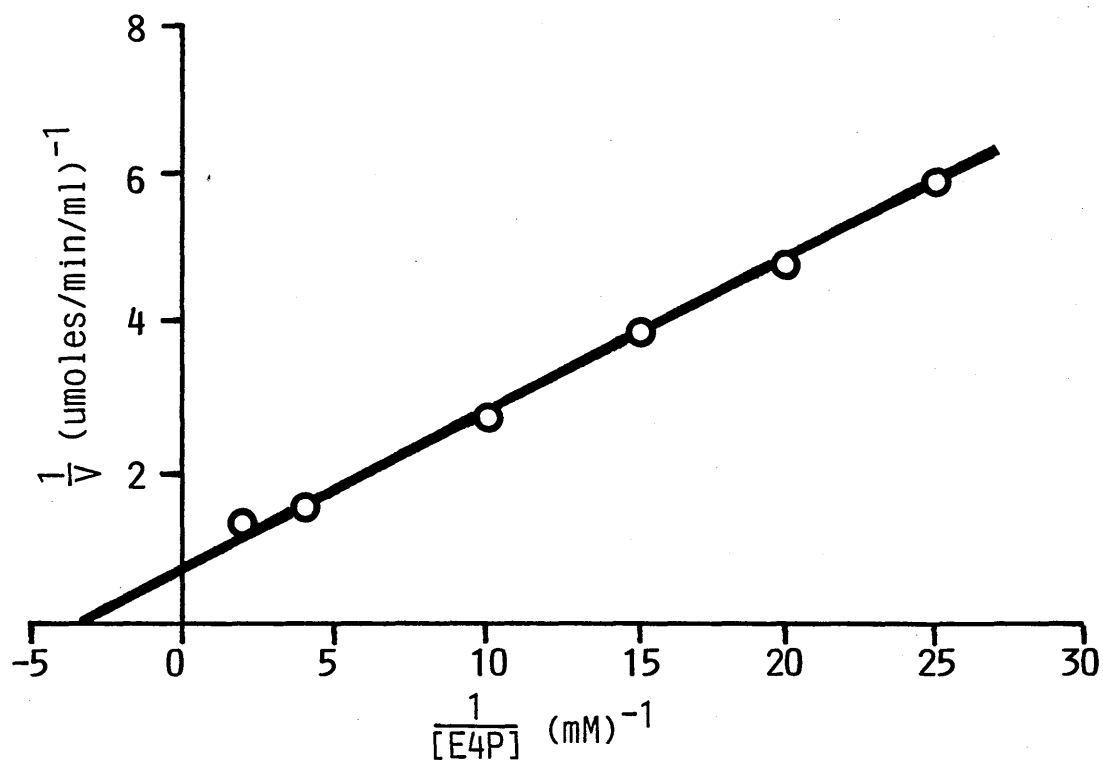


Figure 3.19 Determination of apparent K_m of DAHP synthase for E4P

Double-reciprocal plot of initial velocity against E4P concentrations at 0.5 mM-PEP. DAHP synthase was assayed either by the direct or the coupled continuous assay as described in section 3.10.

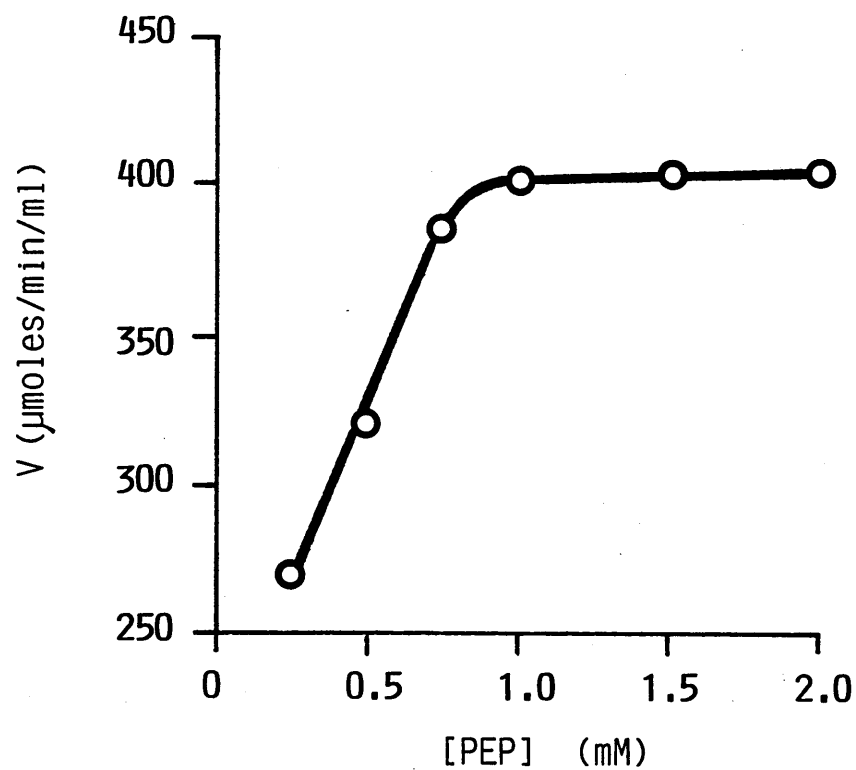


Figure 3.20 The initial DAHP synthase reaction against PEP concentration

DAHP synthase was assayed by the coupled assay method as described in section 3.10 with $[\text{PEP}]$ varied as stated and $[\text{E4P}]$ fixed at 0.5 mM.

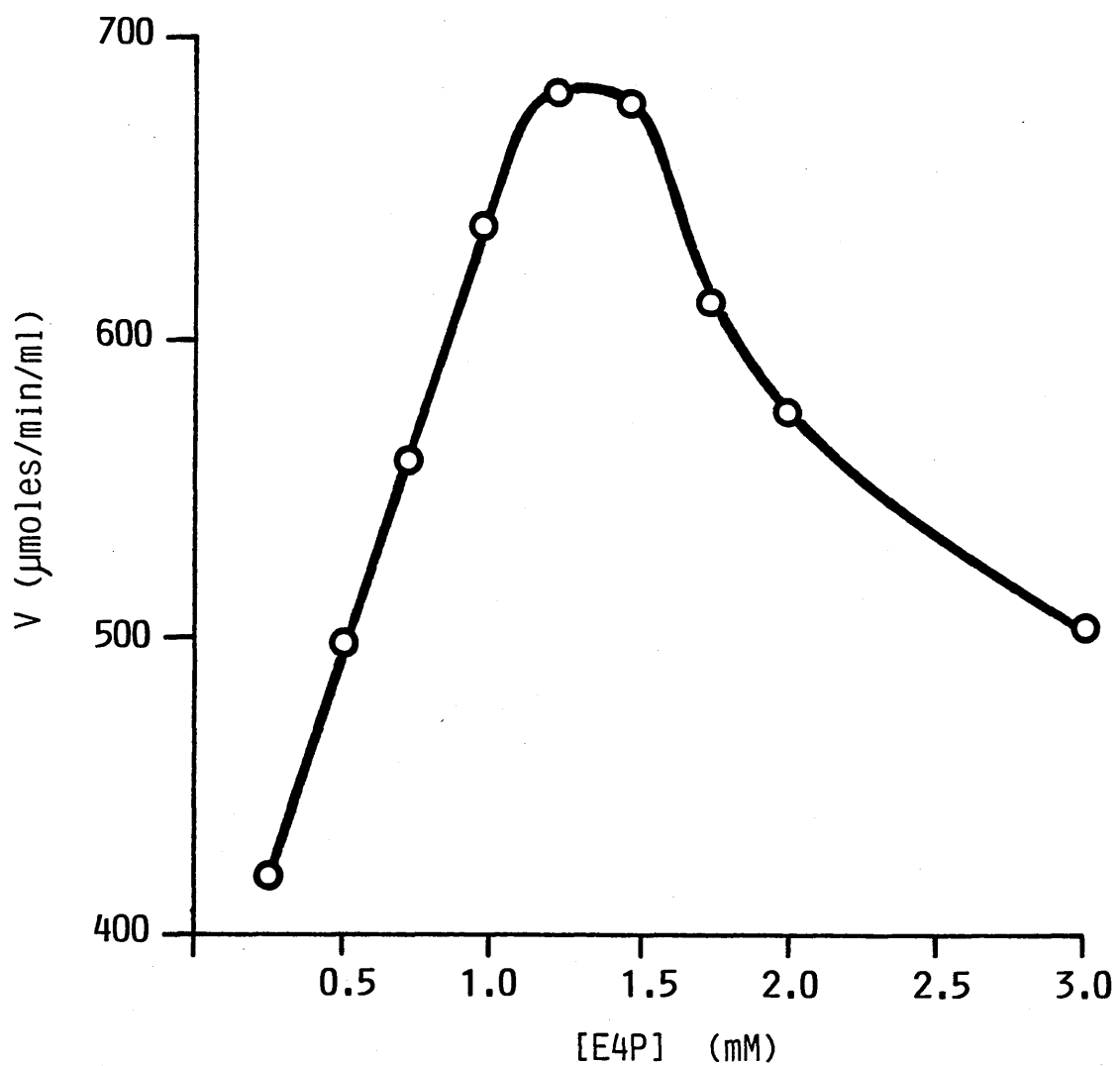


Figure 3.21 The initial DAHP synthase reaction against E4P concentration

DAHP synthase activity was assayed by the direct method as described in section 3.10 with [E4P] varied as stated and [PEP] fixed at 0.5 mM.

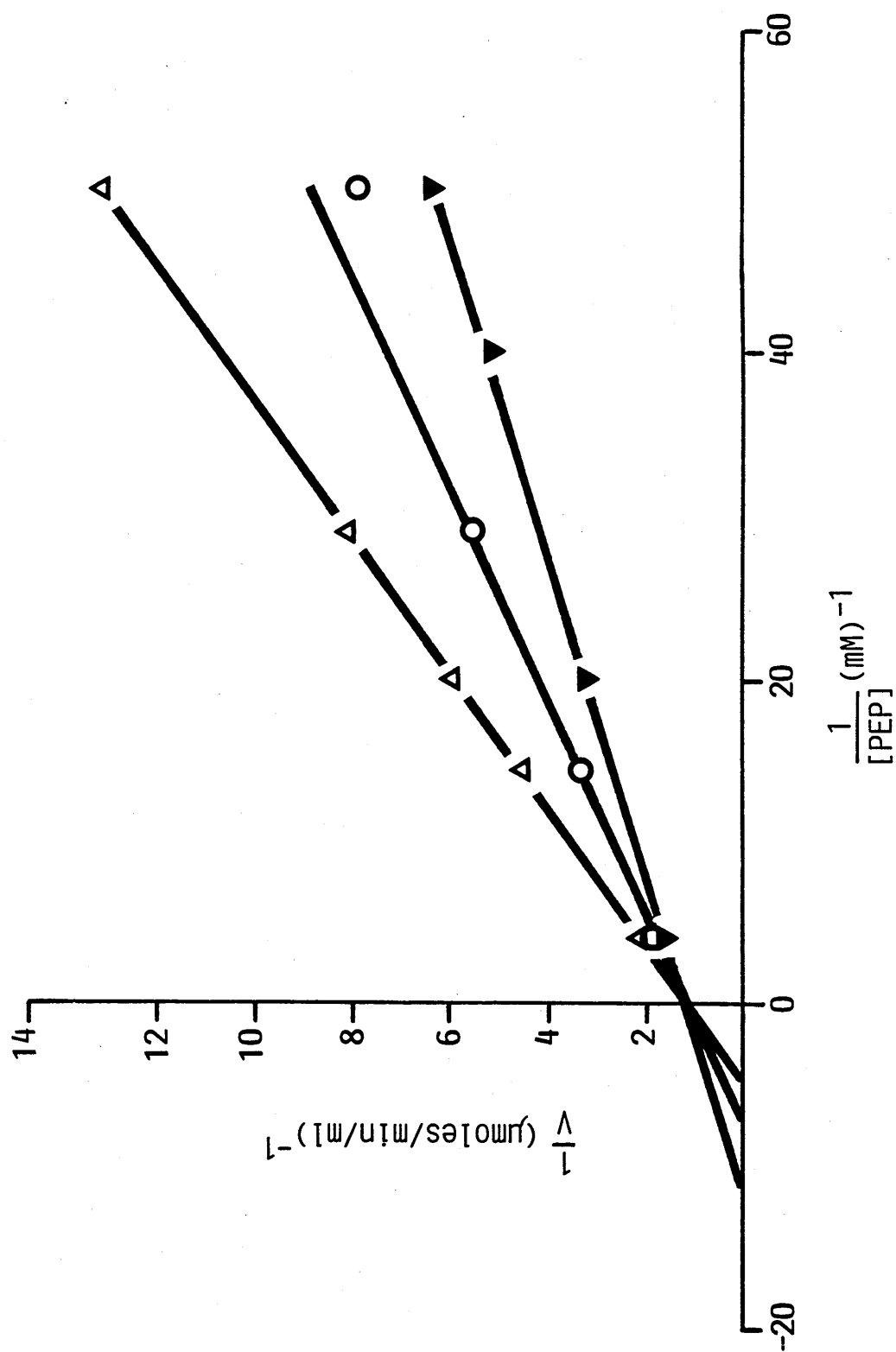


Figure 3.22 Inhibition of the *S.coelicolor* DAHP synthase by E4P

DAHP synthase was assayed by the direct method (section 2.4.1) at the specified substrate concentrations as described in section 3.10.1.

(▼) 1.1 mM-E4P ; (o) 2 mM-E4P ; (Δ) 3 mM-E4P

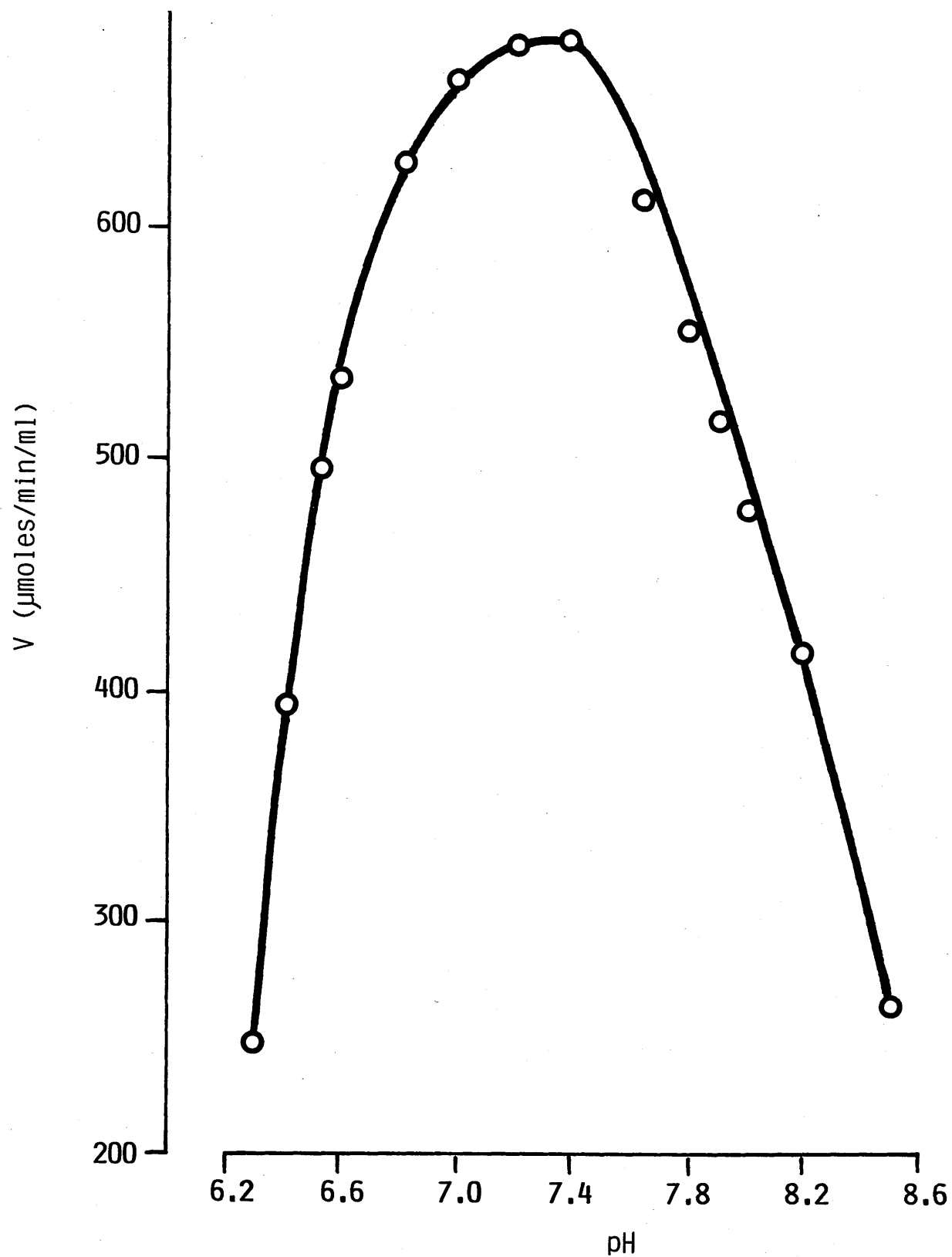


Figure 3.23 pH optima of the *S.coelicolor* DAHP synthase

DAHP synthase was assayed by the direct method as described in section 3.10. The values plotted are the maximal measured at each pH. The curve was fitted by eye.

Table 3.5 Stability of the *S.coelicolor* DAHP synthase under various buffer and pH conditions

Aliquots of the 35-65% $(\text{NH}_4)_2\text{SO}_4$ fraction were dialysed overnight at 4°C against either 50 mM-potassium phosphate, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT, or 50 mM-Tris/HCl, 1.2 mM-PMSF, 3.5 mM-benzamidine, 0.4 mM-DTT at the indicated pH as described in section 3.10.3. Samples were assayed under standard conditions by the coupled assay method (section 2.4.1.(a)). One sample was left untreated and contained $(\text{NH}_4)_2\text{SO}_4$.

| Buffer | pH | Specific Activity (mU/mg) | % Loss in activity |
|-------------------------------|-----|------------------------------|-----------------------|
| potassium phosphate | 6 | 1.8 | 72 |
| | 6.5 | 3.7 | 41 |
| | 7 | 4.1 | 34 |
| | 7.5 | 3.8 | 40 |
| $+(\text{NH}_4)_2\text{SO}_4$ | 7 | 6.3 | 0 |
| Tris/HCl | 7.5 | 0.5 | 92 |
| | 8 | 0.4 | 94 |
| | 8.5 | 0.1 | 98 |

3.10.3 Metal ion dependence of enzyme activity

Inclusion of 5 mM-EDTA in extraction buffer (buffer A) apparently had no effect on DAHP synthase activity levels found in crude cell-free preparations, however overnight dialysis at 40°C of (NH₄)₂SO₄ pellet aliquots against buffer B supplemented with 10 mM-EDTA resulted in almost total inactivation (Table 3.6) of the enzyme. Inclusion of 30 µM-CoCl₂ in the coupled assay mixture resulted in a partial recovery of measured DAHP synthase activity (Table 3.6).

DAHP synthase activity was also lost following overnight dialysis against Tris buffers at various pH values (Table 3.5), possibly as a result of the metal chelating properties of this buffer. Consequently phosphate buffers were employed throughout the purification and EDTA was not used.

3.10.4 Susceptibility to oxidation

Fresh DTT (0.4 mM) was included in all buffers following the observation that inactivated enzyme could be partially reactivated by the addition of this thiol reducing agent.

3.11 Repression and inhibition of the *S.coelicolor* DAHP synthase

Enzymes contributing significantly to the regulation of a pathway are frequently controlled at either the catalytic or allosteric level by product feedback inhibition or at the transcriptional level by repression and induction. The possibility that these control mechanisms influenced the *S.coelicolor* DAHP synthase activity was investigated.

3.11.1 Repression of synthesis of DAHP synthase

S.coelicolor cells were grown on a rich medium (YEME) and cell-free extracts prepared

Table 3.6 Inactivation of *S.coelicolor* DAHP synthase by EDTA

Aliquots of the 35-65% (NH₄)₂SO₄ fraction were dialysed overnight at 4°C against buffer A (Table 3.1) containing EDTA as described in section 3.10.3; as a control, an aliquot was dialysed against buffer A alone. Fractions were assayed under standard conditions by the coupled assay method (section 2.4.1 (a)).

| Incubated with 10mM-EDTA | Assayed with 30 µM-CoCl ₂ | Total Activity (mU) | % Loss in activity |
|-----------------------------|---|------------------------|-----------------------|
| + | + | 100 | 75 |
| + | - | 62 | 85 |
| - | + | 390 | 0 |

and assayed for DAHP synthase activity. Assays could not be performed on this material due to the high background rate but activity could be purified (section 3.2) and assayed at subsequent stages. The specific activity of DAHP synthase, although lower at each stage of the purification (Table 3.7) than that found in extracts purified from minimal medium grown cells, was clearly not fully repressed.

3.11.2 Inhibition of enzyme activity

A range of skikimate pathway end-products and intermediates were evaluated for their ability to inhibit the *S.coelicolor* DAHP synthase (Table 3.8). Only tryptophan was found to elicit any significant effect where under specified conditions inhibition over control activity was up to 67%. (Table 3.8). At concentrations up to 1 mM, tyrosine and phenylalanine had no effect on enzyme activity, and similarly at concentrations up to 0.25 mM, anthranilate and *p*-aminobenzoate gave no inhibitory result. The tryptophan inhibition was saturable with an apparent K_i of 70 μ M (Figure 3.24). This allosteric property was stable in extracts throughout the purification and stable for at least 6 months in purified extracts stored in buffer H at -20°C.

3.12 Storage and stability of the *S.coelicolor* DAHP synthase

Enzyme stored in buffer H for 6 months at -20°C had lost 20% of the original activity with the remaining activity fully sensitive to tryptophan inhibition.

3.13 Discussion

This chapter reports the purification to homogeneity of a tryptophan-sensitive DAHP synthase from the gram-positive actinomycete *Streptomyces coelicolor*. The enzyme was prepared either in a four step procedure yielding μ g amounts of material (Table 3.3) or in a five step protocol capable of providing the mg amounts of protein (Table 3.4) necessary for

Table 3.7 Purification of *S.coelicolor* DAHP synthase from complex medium-grown cells.

The results presented are for a typical purification starting from 40g of *S.coelicolor* cells (section 3.11.1).

| Step | Total Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) | Yield % | Purification (fold) |
|---|--------------------|---------------------|---------------------------|---------|---------------------|
| 1. Crude | 400 | ND | ND | ND | ND |
| 2.(NH ₄) ₂ SO ₄ fractionation | 219 | 350 | 1.6 | 100 | 1 |
| 3. Mono Q chromatography | 16.5 | 308 | 18.5 | 88 | 12 |
| 4. Phenyl Superose chromatography | 0.45 | 100 | 222.2 | 32.5 | 139 |

Table 3.8 Screening of putative inhibitors of *S.coelicolor* DAHP synthase

DAHP synthase was assayed either by the direct or the coupled assay method (section 2.4.1); the assay mixture was modified and contained 50 μ M-PEP, 100 μ M-E4P and the indicated effector concentration as described in section 3.11.2.

| Effector [mM] | Activity (mU/ml) | % Inhibition |
|---------------------------------|---------------------|--------------|
| NONE | 369 | 0 |
| 0.12 mM-tryptophan | 122 | 67 |
| 1.0 mM-tyrosine | 394 | 0 |
| 1.0 mM-phenylalanine | 386 | 0 |
| 0.25mM-anthranilate | 352 | 0 |
| 0.25mM- <i>p</i> -aminobenzoate | 390 | 0 |

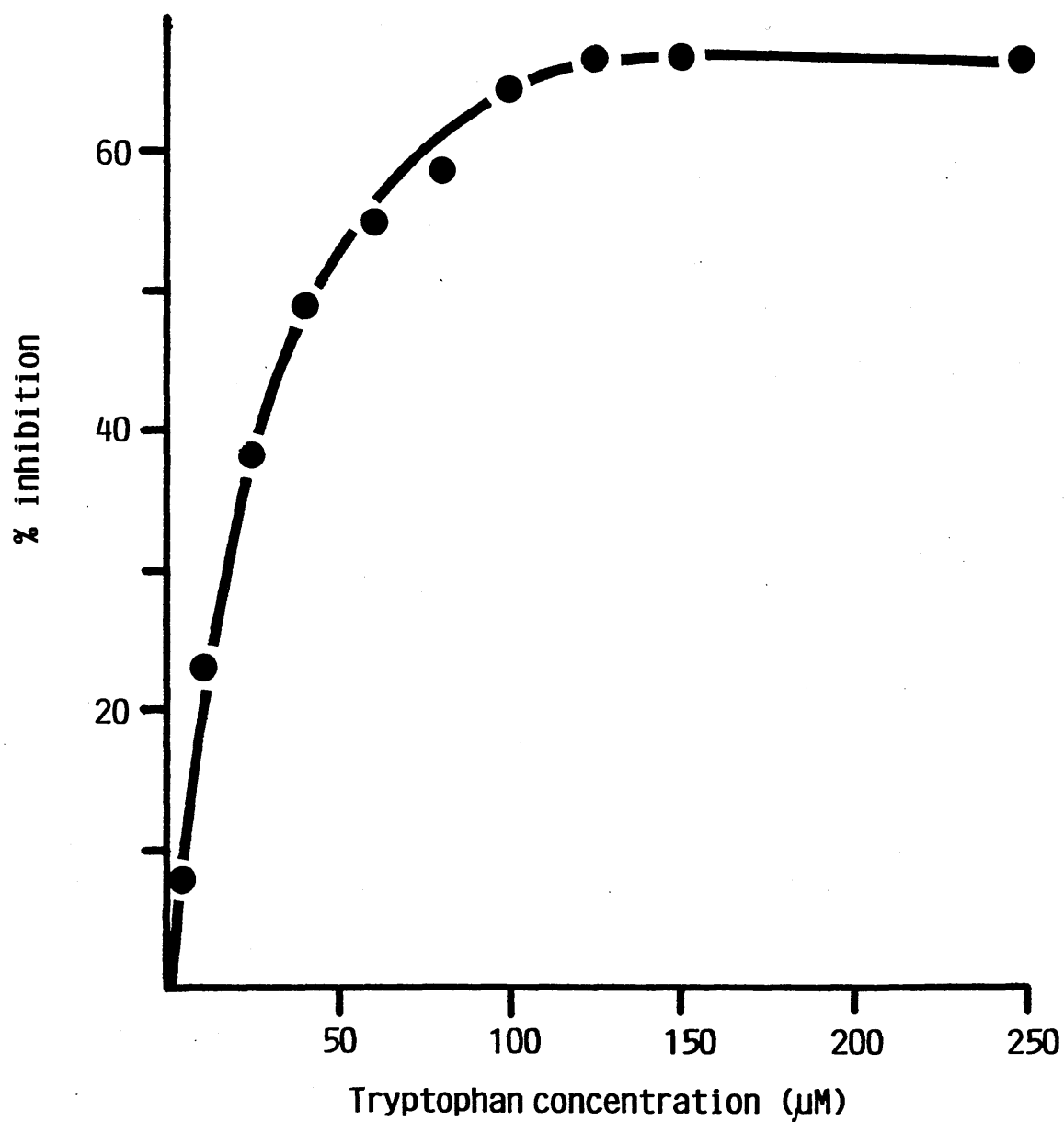


Figure 3.24 Saturation curve of tryptophan inhibition of the *S.coelicolor* DAHP synthase.

DAHP synthase was assayed by the direct method (section 2.4.1) with the addition of between 0 to 250-μM tryptophan as described in section 3.11.2. Assays contained final concentrations of 50 μM-PEP and 100 μM-E4P.

N-terminal amino acid sequence analysis and peptide-generating experiments (Chapter 4).

The activity of the enzyme found in extracts made from cells grown on minimal media was low, typically 3-4 mU/mg protein (Table 3.3). This is similar to levels found in fungi (Nimmo and Coggins, 1981a) but approximately 100 fold less than that found in wild-type *E.coli* (Schoner and Herrmann, 1976). Progress was further hampered by the difficulties in growing *S.coelicolor* in liquid minimal medium and obtaining sufficient biomass with which to begin a purification. The adoption of Hobbs' new minimal medium (Hobbs *et al.*, 1989) as the growth medium gave biomass yields of around 17g wet weight of cells/litre of culture and protein yields of 10mg/g wet weight of cells. This protein yield is around 5 times lower than that found in extracts made from *E.coli* (J.Greene, personal communication).

The purification fold as judged by the final residual activity is 800-fold, with a 10% yield (Table 3.3). However a major loss of activity occurs at the last step, possibly due to much of the remaining metal attached to the enzyme being stripped off upon passage through the Mono Q column in a DTT-containing buffer (DTT has metal-chelating properties ; the Mono Q support matrix may have similar properties). By estimates of protein before and after this step we calculate that the purification factor is nearer 2,000-fold (Table 3.3). The increase in the time taken to complete each step in the large scale purification results in an even poorer yield (Table 3.4) ; with either more metal cofactor being lost earlier in the procedure or the increase in the length of the purification facilitating increased oxidation. Closer analysis of yields from individual steps indicates that cold-room chromatographic procedures and other manipulations undertaken there such as dialysis resulted in the greatest losses in activity. The procedures carried out at room temperature on an f.p.l.c. apparatus gave a far higher recovery of activity. One possible conclusion is that the enzyme is partially cold-labile. These problems could be addressed by the inclusion of some divalent metal cations in all buffers, the use of an alternative reductant lacking chelating properties such as mercaptoethanol, and the purification being carried out at room

temperature.

No evidence was found to indicate the presence of two or more isozymes of DAHP synthase as has been found in numerous other organisms (see section 1.2.2), at no stage were separable activities detected nor was there a dramatic loss in activity indicating the loss of an isozyme. The cloned gene could be used as a tool in a gene disruption experiment to prove conclusively the presence of a single gene encoding a single enzyme. In the two reported partial purifications of the enzyme from two species of streptomycete (Gorisch and Lingens, 1971a ; Lowe and Westlake, 1970) no isoenzymic forms of the activity were detected and it was concluded that a single enzyme was present.

Affinity chromatography has been used successfully in the rapid purification of minute amounts of biological material from a more complex mixture. It has been argued that hydroxylapatite and, more convincingly, phosphocellulose may act as "pseudo" affinity columns in cases where enzymes bind substrates possessing one or more phosphate moieties such as PEP. These two materials have been used successfully in DAHP synthase purification protocols from a variety of organisms:- phosphocellulose in the purification from *N.crassa* (Nimmo and Coggins, 1981a) and from potato (Pinto *et al.*, 1986) ; hydroxylapatite in the isolation from *Nocardia mediteranei* (Tianhui and Chiao, 1989) and from two of the *E.coli* isozymes (Schoner and Herrmann, 1976 ; McCandliss and Herrmann, 1978). In one of these examples that of the tryptophan inhibitable *N.crassa* enzyme, substrate elution from phosphocellulose with PEP containing buffers was the crucial step which facilitated a rapid, high yield purification.

Our attempts to utilize these media as a final purification step did not prove successful. The *S.coelicolor* DAHP synthase failed to bind to phosphocellulose (section 3.8.1(a)) under conditions used previously to bind other PEP utilizing enzymes such as EPSP synthase from *E.coli* (Lewendon and Coggins, 1983) and the tryptophan sensitive DAHP synthase from *N.crassa* (Nimmo and Coggins, 1981a).

The *S.coelicolor* DAHP synthase did bind to hydroxylapatite, but weakly and could not be dislodged by substrate (section 3.8.1(b)). It was concluded that these media were not in this case acting as affinity columns but as general cationic exchangers. If hydroxylapatite was acting as a general cationic exchanger then one might expect another such exchanger to bind the activity at a similar pH and ionic strength. DAHP synthase from *S.coelicolor* failed to bind to the Mono S cationic exchanger under such conditions (section 3.8.3) but did bind to the Mono Q anionic exchanger (section 3.2). Although the mechanism of binding proteins to hydroxylapatite is not fully understood it appears unlikely to bind *S.coelicolor* in a ligand-recognising fashion.

The *S.coelicolor* DAHP synthase appears to be a homodimer with a subunit M_r of 54kDa. This is effectively confirmed by the single *N*-terminal sequence which was obtained for the enzyme (section 4.2). DAHP synthases from a variety of organisms display heterogeneity both in their subunit size and quaternary structure (Table 3.9). It is possible to crudely divide these enzymes into two classes on the basis of subunit size (Table 3.9), with one class displaying an estimated subunit M_r in the range 38-44 kDa (class1) and the other with estimated M_r in the range 52-56 kDa (class 2). Errors in subunit M_r estimation may have broadened these criteria. The smaller class includes the enteric bacteria, yeast, *Acinetobacter* sp., and an actinomycete representative. The larger subunit class includes plant, fungal and *Streptomyces* members, in particular *S.coelicolor* (Table 3.9).

A surprising difference in quaternary structure is observed between the DAHP synthases in both classes. The majority of enzymes in both classes are dimers or tetramers, with evidence that the quaternary structure confers effector sensitivity (de Boer *et al.*, 1989) as in this case dissociated monomers were inhibitor insensitive. However, the existence of at least four monomeric class1 forms (Byng *et al.*, 1985; Paravicini *et al.*, 1989; G.Braus, personal communication, 1991), three of which are sensitive to end-product inhibition, indicates that subunit organisation is not essential in conferring inhibition in all cases.

Table 3.9 Comparison of subunit size and oligomeric structure of DAHP synthases

| Organism | Subunit M _r (kDa) | Native M _r (kDa) | Oligomeric structure | Class | Reference |
|---------------------------------|------------------------------|-----------------------------|----------------------|-------|-------------|
| <i>E.coli</i> | | | | | |
| phenylalanine | 38 (a) | 160 (c,d) | tetramer | 1 | (1), (2) |
| tyrosine | 39 (a) | 80 (c) | dimer | 1 | (3), (4) |
| tryptophan | 39 (a) | 80 (c) | dimer | 1 | (5),(6) |
| <i>A.calcoaceticus</i> | | | | | |
| tyrosine | 43 (c) (?) | 43 (c) | monomer | 1 | (7) |
| unregulated | 45 (c) (?) | 45 (c) | monomer | 1 | (7) |
| <i>S.cerevisiae</i> | | | | | |
| phenylalanine | 42 (a) | 53 (c) | monomer | 1 | (8), (9) |
| tyrosine | 42 (a) | (c) | monomer | 1 | (18) |
| <i>P.aeruginosa</i> | | | | | |
| tyrosine | 35 (?) | 137 (c) | tetramer | 1 | (12) |
| tryptophan | 42 (?) | 175 (c) | tetramer | 1 | (12) |
| <i>N.mediterranei</i> | 35 (b) | 140 (c) | tetramer | 1 | (10) |
| <i>Nocardia</i> sp.239 | 43 (c) | 168 (c) | tetramer | 1 | (11) |
| <i>N.crassa</i> | | | | | |
| tryptophan | 52 (b) | 200 (d) | tetramer | 2 | (13) |
| <i>B.flavum</i> | 55 (b) | 220 (c) | tetramer | 2 | (14) |
| Carrot roots | 53 (b) | 103 (c) | dimer | 2 | (16) |
| Potato | 56 (a) | 110 (c) | dimer | 2 | (17) |
| <i>S.aureofaciens</i> | 55 (?) | 100 (c) | dimer (?) | 2 | (15) |
| <i>Streptomyces</i> sp.3022a | 55 (?) | ~100 (c) | dimer (?) | 2 | (19) |
| <i>S.rimosus</i> | 54 (b) | ND | (?) | 2 | (23) |
| <i>S.coelicolor</i> | 55 (b) | 107 (c) | dimer | 2 | section 3.9 |

Notes on Table 3.9

(a) determined from nucleotide sequence

(b) SDS PAGE analysis

(c) evaluated by gel filtration chromatography

(d) sedimentation-equilibrium centrifugation

(?) inferred from data, but not conclusively known

The references are listed below Table 3.11

In the majority of cases substrate kinetics have obeyed Michaelis-Menten behaviour. Preliminary experiments indicate co-operativity of substrate binding with the tetrameric *E.coli* phenylalanine-sensitive isozyme (C.Stephens, personal communication, 1990) and that this tetrameric enzyme may be truly allosteric in its substrate kinetics and regulatory behaviour. There is one other example of homotropic behaviour, that of the DAHP synthase from *Brevibacterium flavum* (Sugimoto and Shiio, 1980), which was shown conclusively to be a typical allosteric enzyme with homotropic cooperativity of binding of one substrate, PEP.

The steady-state kinetic parameters of the *S.coelicolor* DAHP synthase are compared with those evaluated from other organisms in Table 3.10. Kinetic values determined from purified, highly active, often over-produced enzyme are listed in rows one to five. The more sensitive direct PEP utilization assay (section 2.4.1(c)) was also employed in these studies. For each substrate the values are at least one order of magnitude lower than those determined from cruder, less active and often less stable preparations by other assay methods (rows 7-13). However, in general, most enzymes possess a higher affinity for PEP as opposed to E4P. There appears to be no correlation between the class of the enzyme and the K_M values. This may reflect the varying quality of the enzyme preparations and data, especially when high K_M 's are reported.

The *S.coelicolor* DAHP synthase displayed a narrow activity pH optimum between 7 and 7.4 ; the stability of the activity was preserved best in buffers of pH 7. Table 3.10 lists the pH optima of a number of microbial DAHP synthases which also lie within this interval. In plants an isozyme with a different pH profile has been described (section 1.2.2.1 (d)).

DAHP synthases from a variety of organisms are susceptible to inactivation by metal chelating agents such as EDTA (Staub and Denes, 1969a,b ; Gorisch and Lingens, 1971a ; Simpson and Davidson, 1976 ; Nimmo and Coggins, 1981a ; Paravicini *et al.*, 1989) and this inactivation can be protected against by bivalent metal ions and by substrate, such as

Table 3.10 Kinetic parameters of microbial DAHP synthase enzymes

| Organism | pH optimum | K _m PEP(μM) | K _m E4P(μM) | Enzyme preparation | Assay used | Reference |
|-------------------------------------|------------|------------------------|------------------------|--------------------|------------|--------------|
| <i>E.coli</i> | | | | | | |
| 1. phenylalanine | 6.5-7.0 | ~5.8 | ND | PS | D | (1) |
| 2. tyrosine | 7.0 | 5.8 | 96.5 | PS | D | (3) |
| 3. tryptophan | 7.0 | 9 | 50 | OPS | D | (20) |
| <i>S.cerevisiae</i> | | | | | | |
| 4. phenylalanine | 6.8 | 18 | 130 | OPS | D | (9) |
| <i>N.crassa</i> | | | | | | |
| 5. tryptophan | 6.5-7.5 | 12 | 2.7 | PS | D | (13) |
| 6. <i>S.coelicolor</i> | 7.0-7.4 | 100 | 285 | PPS(400) | DC | section 3.10 |
| 7. <i>S.aureofaciens</i> | 7.0-7.5 | 300 | 160 | PPS(14) | CH | (15) |
| 8. <i>Nocardia</i> sp.239 | 7.0-7.5 | 45 | 370 | PPU(20) | CH | (11) |
| 9. <i>N.mediterranei</i> | 7.2 | 400 | 250 | PS(120) | CH | (10) |
| <i>P.aeruginosa</i> | | | | | | |
| 10. tyrosine | 6.8 | 1000 | 1000 | PPS(ND) | CH | (12) |
| 11. tryptophan | 7.2 | 1110 | 560 | PPS(ND) | CH | (12) |
| 12. <i>S.antibioticus</i> | 6.8 | 150 | 150 | NHS(ND) | CH | (21) |
| 13. <i>Streptomyces</i> sp.3022a | 7.2 | 100 | 100 | PPS(ND) | CH | (19) |

Notes on Table 3.10

O - overproduced

PP- partially purified (fold)

NH- ammonium sulphate fraction

CH -stopped chemical assay

P- purified to homogeneity

U -unstable

D -direct PEP utilization assay

S- stable

ND- not determined

C -coupled assay

The references are listed below Table 3.11

PEP. The identity of the metal is thought to be Fe^{2+} in the *E.coli* isozymes (McCandliss and Herrmann, 1978 ; Ray and Bauerle, 1991) and the phenylalanine isozyme of *Saccharomyces cerevisiae* (Paravicini *et al.*, 1989), although the former finding is disputed for the tyrosine-sensitive *E.coli* isozyme which may be a copper metalloenzyme (Baasov and Knowles, 1989). The *S.coelicolor* DAHP synthase was similarly inactivated by EDTA but no protection studies were undertaken.

The most likely explanation for the ubiquitous presence of metal in DAHP synthases is that they form part of the group of metalloaldolases, designated class II (reviewed in Lai and Horecker, 1972). These aldolases use a divalent metal cation to stabilise or promote the formation of a carbanion group on the substrate which is essential for reactivity. Class II aldolases are characterised by their inhibition by chelating agents and exhibit sharp pH optima (Rutter, 1964).

In common with the majority of successful purifications of DAHP synthase from different organisms that from *S.coelicolor* included DTT in buffers in order to prevent the oxidation of sulfhydryl groups and to preserve activity. The two notable exceptions are the purification procedures of the *E.coli* phenylalanine and tyrosine sensitive isozymes (Schoner and Herrmann, 1976 ; McCandliss *et al.*, 1978) which did not include a reductant, however the presence of PEP in all buffers also appears to protect active-site sulfhydryl groups from oxidation, as its removal results in inactivation.

Many DAHP synthases can be inhibited by thiol-modifying reagents (Moldovanyi and Denes, 1968 ; Staub and Denes, 1969a,b ; Gorisch and Lingens, 1971a ; Huisman and Kosuge, 1974 ; Shio and Sugimoto, 1979a,b ; Ray and Bauerle, 1991) with PEP but not E4P providing protection against inactivation. It was concluded (Staub and Denes, 1969a,b) that DTNB-sensitive sulfhydryl groups were implicated either in the PEP binding site or the catalytic site. Sugimoto and Shio (1979a,b) concluded that in *Brevibacterium flavum* a single sulfhydryl group per mol of subunit was essential for activity, whereas for

the *E.coli* phenylalanine-sensitive isozyme two groups per subunit monomer were thought to be essential (C.Stephens, personal communication, 1990), with the identity of these groups currently being determined.

Ganem (1978) has suggested an enzyme-bound sulphur-containing intermediate to explain these modification experiments and other isotope labelling data (discussed in section 1.2.1). Furthermore, the possibility that the sulfhydryl group is involved in co-ordinating the metal ion cofactor remains plausible as metal ions are able to partially protect against DTNB inactivation (C.Stephens, personal communication, 1990) of the *E.coli* phenylalanine-sensitive isozyme.

Enzyme catalysed reactions early in a biosynthetic pathway are likely targets for regulation of the metabolic flow. DAHP synthase is a target for a variety of forms of feedback inhibition in different microorganisms and repression control is also found in some organisms (section 1.2.3).

The *S.coelicolor* DAHP synthase activity was found to be inhibited solely by tryptophan with an apparent K_i of $70\mu\text{M}$ under the assay conditions employed; the maximal inhibition under these conditions was 67% of control. Superfamily-B prokaryotes (excepting the Group V pseudomonads), fungi and yeast possess two or more isoenzymic forms of DAHP synthase (Jensen and Nasser, 1968 ; Lingens *et al.*, 1966 ; Jensen and Ahmad, 1988). Gram-positive bacteria of the *Actinomycetales*, including *Nocardia* and *Streptomyces* species possess a single DAHP synthase (Lowe and Westlake, 1970 ; Gorisch and Lingens, 1971a,b ; de Boer *et al.*, 1989 ; Tianhui and Chiao, 1989) which, with one exception, is inhibited only by tryptophan at physiologically relevant effector concentrations. The DAHP synthase of *Streptomyces* sp.3022a was found to be aromatic inhibitor insensitive (Lowe and Westlake, 1970), but this study employed a commercial production strain which may have possessed a mutant deregulated phenotype.

The effect of tryptophan on the three streptomycete DAHP synthases is summarised in

Table 3.11 and compared with another actinomycete representative and the *E.coli* tryptophan isozyme. The streptomycete enzymes are comparatively poorly inhibited by tryptophan and, in common with other tryptophan sensitive species, usually only partial inhibition is observed. The inability of tryptophan to elicit total inhibition is logical in a single isozyme system as a surfeit of this single amino acid end-product would prevent the synthesis of any other products if total inhibition occurred.

DAHP synthase activity was found in *S.coelicolor* cells grown on a rich medium and this activity could be purified in an analogous fashion to material derived from minimal medium grown cells (Table 3.7). No evidence of repression control has been found for DAHP synthase and other shikimate pathway enzymes in the *Actinomycetales* (Lowe and Westlake, 1970 ; Gorisch and Lingens, 1971b ; de Boer *et al.*, 1989), although one group (Murphy and Katz, 1980) claimed the activity was repressible, but other explanations of their results such as specific degradation were not considered and the experimental method employed may not have been satisfactory. One example of feedback activation by secondary products on primary metabolism in *Penicillium cyclopium*, in particular the aromatic amino acid biosynthesis enzymes DAHP synthase, chorismate mutase and anthranilate synthase has been reported (Roos and Schmauder, 1989). Here, the addition of secondary product to cultures resulted in stimulation in the synthesis of these enzymes. It would be of great interest to investigate the possibility of a similar mechanism existing in an aromatic containing antibiotic producing streptomycete. In contrast, in the enteric bacteria and yeast, exquisitely sensitive repression control systems have been described (section 1.2.3).

From the evidence outlined above it would appear that the metabolic control of the shikimate pathway in *S.coelicolor* follows an exo-orientated pattern as described by Jensen and Hall (1982). In summary, microorganisms can be classified into two groups according to the control system features of their respective amino acid biosynthetic machinery. One group, where control is orientated to exogeneous substrates, display

Table 3.11 Comparison of selected tryptophan inhibitable microbial DAHP synthases

| Organism | Maximal inhibition (%) | Saturating tryptophan (μM) | Apparent K_i (μM) | [substrate] PEP E4P | | Reference |
|---------------------------|------------------------|---|----------------------------------|------------------------|-------------------|--------------|
| <i>S.coelicolor</i> | 67 | 120 | 70 | $1/2K_m$ | $1/2K_m$ | section 3.11 |
| <i>S.antibioticus</i> | 65 | 100 | 55 | $1/2K_m$ | $1/2K_m$ | (21) |
| <i>S.aureofaciens</i> | 65 | 200 | 110 | $1\frac{1}{2}K_m$ | $1\frac{1}{2}K_m$ | (15) |
| | Maximal inhibition(%) | | K_i (μM) | | | |
| <i>Nocardia</i> sp.239 | 94 | | 3 | | | (11) |
| <i>E.coli</i> | 60 | | 2 | | | (22), (20) |

References cited in Tables 3.9, 3.10 and 3.11

- | | |
|---------------------------------------|---|
| (1) McCandliss and Herrmann, (1978) | (2) Davies and Davidson, (1982) |
| (3) Schoner and Herrmann, (1976) | (4) Schultz <i>et al.</i> , (1984) |
| (5) Poling <i>et al.</i> , (1981) | (6) Ray <i>et al.</i> , (1988) |
| (7) Byng <i>et al.</i> , (1985) | (8) Paravicini <i>et al.</i> , (1988) |
| (9) Paravicini <i>et al.</i> , (1989) | (10) Tianhui and Chiao, (1989) |
| (11) de Boer <i>et al.</i> , (1989) | (12) Whitaker <i>et al.</i> , (1982) |
| (13) Nimmo and Coggins, (1981b) | (14) Sugimoto and Shiio, (1980) |
| (15) Gorisch and Lings, (1971a,b) | (16) Pinto <i>et al.</i> , (1986) |
| (17) Dyer <i>et al.</i> , (1990) | (18) G.Braus, personal communication (1991) |
| (19) Lowe and Westlake, (1971) | (20) Ray and Bauerle, (1991) |
| (21) Murphy and Katz, (1980) | (22) Pittard and Gibson, (1970) |
| (23) Stuart, (1990) | |

complex allosteric regulation both early and at branchpoints of the pathway, this early control being exerted by at least two different terminal end-products, and repression control by amino acid end products. The other, where control is orientated to endogeneous products, frequently exhibit poorer early control governed by a single end product, limited terminal branchlet regulation and no repression control.

These contrasting control strategies were rationalised with respect to an organisms individual ecological niche. In *E.coli*, which undergoes periods of nutrient excess then limitation, competitive success is reliant upon combining efficiency of amino acid biosynthesis during starvation with prudent use of exogeneous substrates upon nutrient excess; consequently they possess biosynthetic machinery highly responsive to exogeneous amino acids. Other organisms, such as *S.coelicolor* which scavenge and may exist in a near-permanent state of nutrient limitation, respond poorly to exogeneous amino acids and have a constitutive biosynthetic capacity. However, exquisite transcriptional control does exist in *S.coelicolor* which has reserved this metabolically expensive mechanism for its outstanding metabolic specialities, antibiotic biosynthesis and colony differentiation (Buttner *et al.*, 1988 ; Chater, 1989 ; section 1.10.1). With regard to the goal of over-producing aromatic precursors for antibiotic production, this simpler endo-oriented regulatory arrangement would appear to facilitate this objective.

We have reported the inhibitory effects of high E4P concentrations on the *S.coelicolor* DAHP synthase, furthermore high concentrations of other sugar phosphates have been found inhibitory (Lowe and Westlake, 1970 ; Tianhui and Chiao, 1989). Inhibition by both substrates at high concentrations has been described (Nimmo and Coggins, 1981a,b) but more commonly E4P alone exhibits this phenomenon, with this inhibition competitive with respect to PEP. One explanation of these results is that assuming PEP binds first (as has been shown, discussed in section 1.2.1) and this binding is rate limiting to catalysis then excess E4P could compete with PEP for binding at a single site and consequently inhibit catalysis. The other sugar phosphates could act in an analagous fashion.

Chapter 4 Primary structure analysis of DAHP synthase
from *Streptomyces coelicolor*

4.1 Introduction

This chapter describes the determination of the *N*-terminal sequence of the *S.coelicolor* DAHP synthase and the sequences of some peptides derived from the intact polypeptide by digestion with proteases. These sequences were used to design two synthetic homologous oligonucleotide probes. Primary sequence comparisons were carried out between the *E.coli* isoenzymes and the one cloned *S.cerevisiae* isoenzyme and a consensus heterologous oligonucleotide designed which was complementary to a putative active site sequence.

4.2 *N*-terminal sequence of the *S.coelicolor* DAHP synthase

The *N*-terminal amino acid sequence of the *S.coelicolor* DAHP synthase was determined by automatic sequencing of the intact protein as described in Materials and Methods (sections 2.7.3 and 2.7.4). The yield of each residue recovered from each cycle of the sequence and the derived sequence is shown in Figure 4.1. The *N*-terminal amino acid was found to be proline indicating that this region has been subjected to post-translational modification to remove the expected methionine residue. The first twenty-six amino acids were identified unambiguously.

4.3 Preparation of peptides from the *S.coelicolor* DAHP synthase

Direct *N*-terminal sequencing was initially unsuccessful when attempted with intact protein prepared for sequencing by exhaustive dialysis against ammonium bicarbonate buffer.

Amino acid sequence data was obtained by sequencing peptides generated from a complete clostripain and a complete tryptic digest of the *S.coelicolor* DAHP synthase as described in Materials and Methods (section 2.7.2). Clostripain is a thiol protease which cleaves on the

Figure 4.1 The *N*-terminal amino acid sequence of the *S.coelicolor* DAHP synthase

Approximately one nanomole of DAHP synthase was sequenced directly following purification and desalting by reverse phase h.p.l.c.

| Sequence number | PTH-amino acid | p moles recovered |
|-----------------|----------------|-------------------|
| 1 | Pro | 976 |
| 2 | Glu | 324 |
| 3 | Asn | 434 |
| 4 | Asn | 602 |
| 5 | Gln | 435 |
| 6 | Lys | 700 |
| 7 | Pro | 740 |
| 8 | Leu | 477 |
| 9 | Thr | 249 |
| 10 | Thr | 342 |
| 11 | Val | 418 |
| 12 | Ala | 580 |
| 13 | Gly | 491 |
| 14 | Ala | 656 |
| 15 | Pro | 445 |
| 16 | Val | 292 |
| 17 | Pro | 557 |
| 18 | Asp | 121 |
| 19 | Asn | 167 |
| 20 | Gln | 152 |
| 21 | Asn | 239 |
| 22 | Ser | 67 |
| 23 | Leu | 242 |
| 24 | Thr | 95 |
| 25 | Ser | 69 |
| 26 | Gly | 290 |

C-terminal side of arginyl residues and to a much lesser extent on the C-terminal side of lysyl residues upon prolonged incubation. Trypsin is a serine protease which also cleaves on the C-terminal side of arginyl and lysyl residues. The lysyl cleavage reaction can be eliminated in both cases by succinylation of the lysyl residues. Two peptides generated by clostripain action and two peptides generated by trypsin were purified and sequenced.

4.3.1 h.p.l.c. separation of peptides produced by a clostripain digest of the *S.coelicolor* DAHP synthase

The peptides generated by the action of clostripain on the *S.coelicolor* DAHP synthase were separated by reverse phase chromatography on a Waters μ Bondapak C18 column attached to a Beckman h.p.l.c. as described in Materials and Methods (section 2.1.4). The material eluting from the column was monitored at 220 nm and several discrete peaks collected by hand, labelled and despatched for automatic pulsed liquid-phase sequencing (Figure 4.2).

4.3.2 h.p.l.c. separation of peptides produced by a tryptic digest of the *S.coelicolor* DAHP synthase

The peptides derived from the tryptic digest of the *S.coelicolor* DAHP synthase were separated by reverse phase chromatography as above (section 4.3.1). Peaks numbered one to eight were collected and sent to be sequenced (Figure 4.3).

Figure 4.2 h.p.l.c. purification of peptides generated by a clostripain digest of the *S.coelicolor* DAHP synthase

A Waters μ Bondapak C18 column was equilibrated in H₂O/ TFA and developed with an acetonitrile gradient in TFA which resolved several peaks. The peaks numbered 1-7 were collected manually, lyophilized and despatched for sequencing.

Flow rate was 0.5ml/min.

Gradient : 0-40% (v/v) CH₃CN in 40 min.

40-60% (v/v) CH₃CN in 10 min.

60% (v/v) CH₃CN for 5 min.

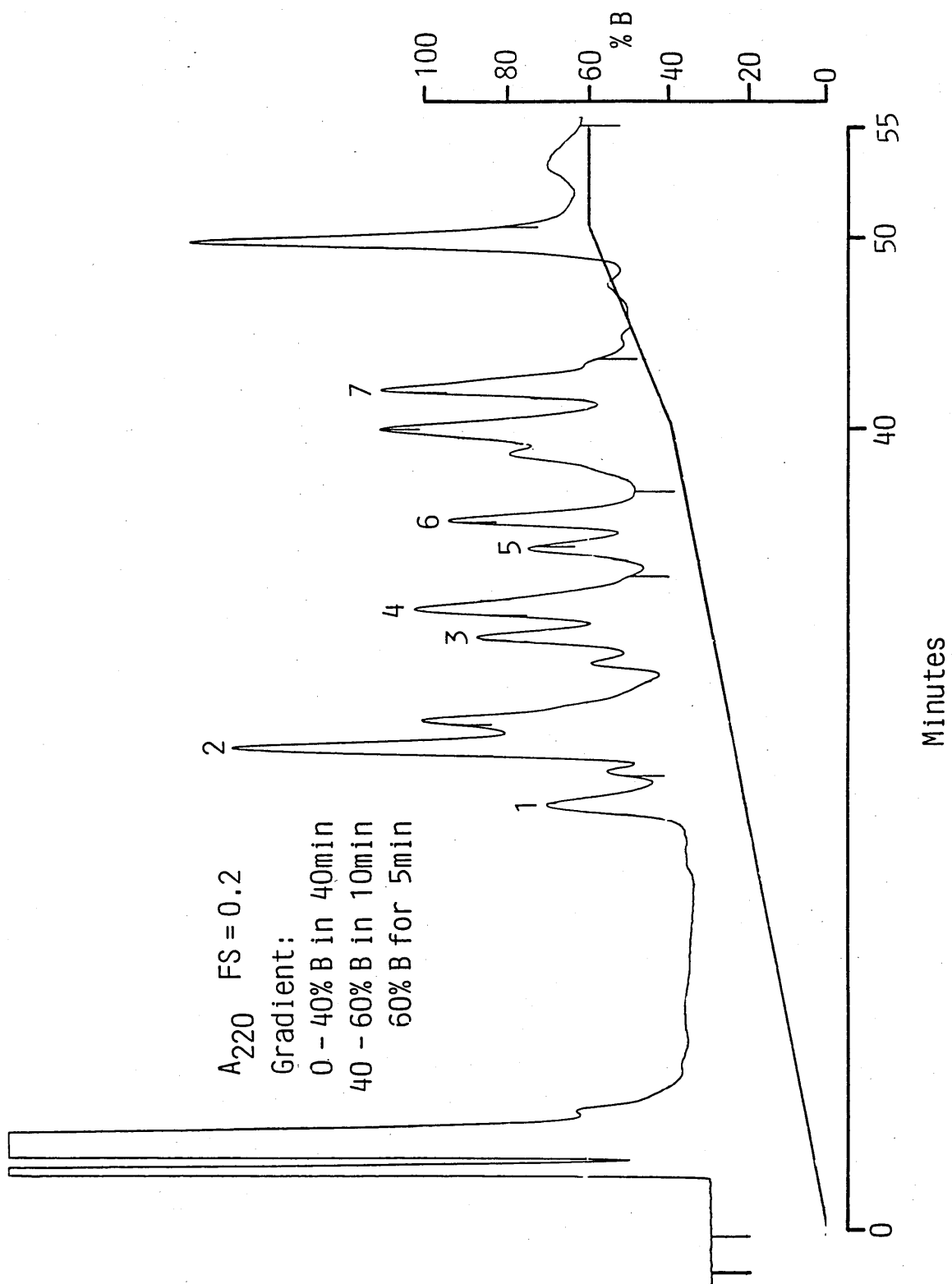


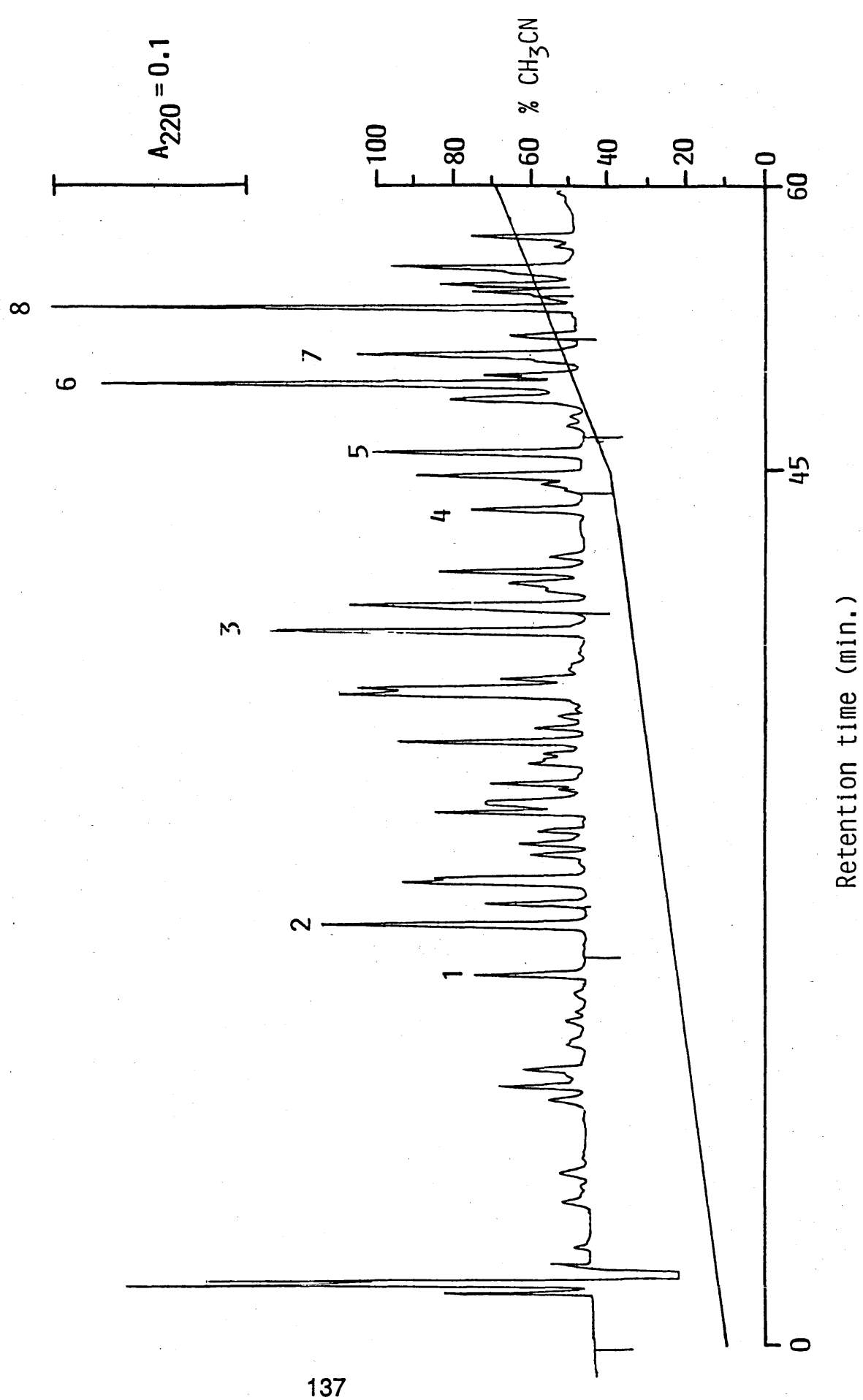
Figure 4.3 h.p.l.c. purification of peptides generated by a tryptic digest of the *S.coelicolor* DAHP synthase

A Waters C18 μ Bondapak C18 column was equilibrated in H₂O/TFA and an acetonitrile gradient developed. The peaks numbered 1-8 were collected manually, concentrated by rotary evaporation, and sent for sequencing.

Flow rate was 0.5ml/min.

Gradient : 0-40% (v/v) CH₃CN in 45 min.

40-70% (v/v) CH₃CN in 15 min.



4.4 Sequencing of peptides

4.4.1 Sequencing of clostripain peptides

Seven peptide peaks were selected from the h.p.l.c. elution profile. Two of these peptides, labelled 1 and 7 on Figure 4.3 were sequenced as described in Materials and Methods (section 2.7.5). The remaining samples comprised mixtures of peptides and unambiguous sequence determination was not possible. The derived sequences are shown in Figure 4.4.

4.4.2 Sequencing of tryptic peptides

Eight peptide peaks were selected from the h.p.l.c. elution profile. Two of these peptides, numbered 1 and 7 on Figure 4.3 were sequenced as in Materials and Methods (section 2.7.4). Samples 5 and 6 were shown to be mixtures of two peptides, but upon further purification too little material was left for sequencing. The yields of residues recovered from each cycle of the sequencer for each of the peptides are shown along with the derived amino acid sequences in Figure 4.5.

4.5 Amino acid composition of the *S.coelicolor* DAHP synthase

Amino acid composition analysis was carried out as described in Materials and Methods (section 2.7.6). Table 4.1 shows the amino acid composition of DAHP synthase from *S.coelicolor*.

Figure 4.4 The amino acid sequence of clostripain peptides 1 and 7

Peptide 1 Val-Phe-Ser-Glu-Ile-Gly-?
 1 6

[illegible]

Figure 4.5 Sequence of tryptic peptides

The yields of PTH amino acids during sequencing of tryptic peptides 1 and 7. The initial amount of peptide sequencing was 0.5 and 1 nmoles, respectively.

| Peptide 1 | | | Peptide 7 | | |
|--------------|----------------|------------------|--------------|----------------|------------------|
| Cycle number | PTH-amino acid | pmoles recovered | Cycle number | PTH-amino acid | pmoles recovered |
| 1 | Phe | 300 | 1 | ? | ? |
| 2 | Asp | 33 | 2 | Pro | 540 |
| 3 | Leu | 60 | 3 | Met | 749 |
| 4 | Arg | 19 | 4 | Leu | 733 |
| | | | 5 | Leu | 613 |
| | | | 6 | Gln | 278 |
| | | | 7 | Asp | 68 |
| | | | 8 | Val | 331 |
| | | | 9 | ? | ? |

The sequence of peptide 7 reported in this table confirmed the earlier sequence in Figure 4.4.

Table 4.1 The amino acid composition of *S.coelicolor* DAHP synthase

| Amino acid | % composition |
|------------|---------------|
| Asx | 15.2 |
| Glx | 11.2 |
| Ser | 4.2 |
| Gly | 8.8 |
| His | 3.6 |
| Arg | 8.1 |
| Thr | 4.8 |
| Ala | 10 |
| Pro | 6.1 |
| Tyr | 3 |
| Val | 5.5 |
| Ile | 3.3 |
| Leu | 5.8 |
| Phe | 6.1 |
| Lys | 4.2 |

These data are averages of two runs on two different analysers (section 2.7.6). The conditions employed for acid hydrolysis destroy tryptophan, and sample was not pre-treated with performic acid to allow identification of cysteine and methionine residues and the analyses of these amino acids are excluded. The values are therefore expressed as the percentages of the amino acids determined, with tryptophan, cysteine and methionine arbitrarily assigned a value of 0%.

4.6 Design of homologous and heterologous oligonucleotide probes

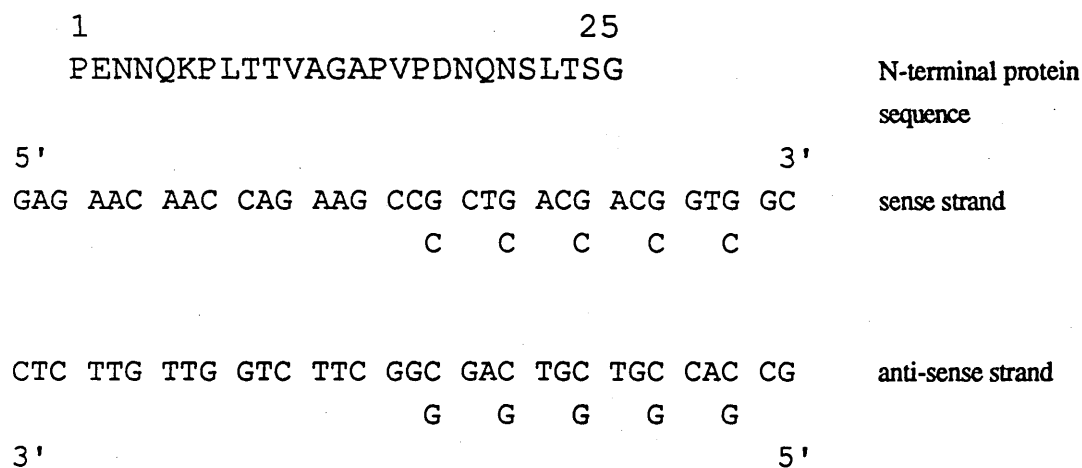
Oligonucleotide probes have been extensively used in "reverse genetics", to allow the isolation of the gene coding a protein of known sequence. Probes are designed from the potential coding sequences of a peptide fragment of known sequence. Recent advances in automated protein microsequencing has made it feasible to isolate genes encoding very minor polypeptide species. Mixtures of oligonucleotides are employed designed against sequences with minimum codon degeneracy, such as those flanking methionine or tryptophan residues. Other least ambiguous codons represent the amino acids asparagine, aspartate, cysteine, histidine, phenylalanine, tyrosine, glutamate, glutamine and lysine. Furthermore, less degenerate oligonucleotides can be designed when a degree of codon preference exists in a given species. For example, within protein coding regions of DNA of *Streptomyces* sp., DNA has a GC content of over 70% and in the third position of any codon there is a 90% chance that the base is guanine or cytosine (Hopwood *et al.*, 1987).

4.6.1 Design of a synthetic oligonucleotide corresponding to N-terminal amino acid sequence

An oligonucleotide probe (32 mer, five degeneracies) was designed corresponding to the sequence of residues 2-12 of *S.coelicolor* DAHP synthase using the known streptomycete codon usage (Hopwood *et al.*, 1987). The anti-sense oligonucleotide was constructed to facilitate mRNA analysis and is shown (Figure 4.6).

Figure 4.6 The design of the oligonucleotide against the *S.coelicolor* DAHP synthase N-terminus

The rationale for the probe design as described in section 4.6.1 is illustrated. The amino acid sequence is shown with the corresponding codon favoured in *Streptomyces* sp. ; if two codons are equally favoured, a degeneracy is introduced in the third base position of the codon. The complementary anti-sense sequence is then derived and the oligonucleotide probe synthesised.



4.6.2 Design of a synthetic oligonucleotide corresponding to internal peptide sequence

An oligonucleotide probe was designed corresponding to the sequence of residues 2-10 of a common tryptic/clostripain peptide (24 mer, 4 degeneracies) and the complementary anti-sense sequence synthesized, see Figure 4.7.

4.6.3 Design of an oligonucleotide corresponding to a putative active site sequence

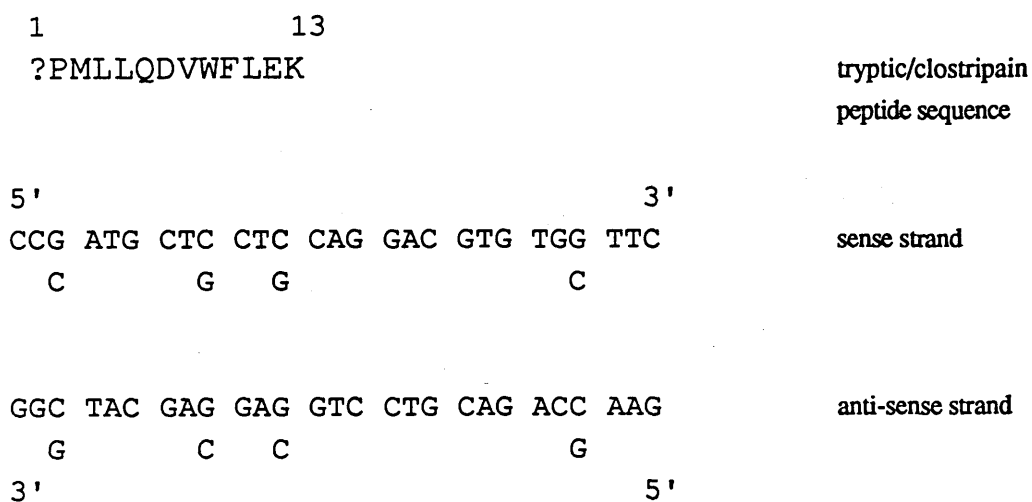
Mutational analysis of the *E.coli* tryptophan sensitive isozyme has revealed putative active site residues (Ray *et al.*, 1988) which lie within two stretches of highly conserved protein sequence which are found in all three *E.coli* isozymes and the two cloned *S.cerevisiae* isozymes (Figure 4.8). Employing the rules of streptomycete codon bias an oligonucleotide probe was designed corresponding to this stretch of 16 conserved amino acids and is shown (47 mer, 8 degeneracies) in Figure 4.9.

4.7 Alignment of *S.coelicolor* DAHP synthase amino acid sequences with the protein sequence data base

WordSearch, Bestfit and Segments programmes were used to carry out comparisons of the *S.coelicolor* sequences with the protein sequence data base as described in Materials and Methods (section 2.22).

Figure 4.7 The design of the oligonucleotide against internal peptide sequence from the *S.coelicolor* DAHP synthase

The rationale for the probe design as described in section 4.6.2 is illustrated. The amino acid sequence is shown with the corresponding codon favoured in *Streptomyces* sp. ; if two codons are equally favoured, a degeneracy is introduced in the third base position of the codon. The complementary anti-sense sequence is then derived and the oligonucleotide probe synthesised.



```

1                                     50
Aro3 .....MFIK NDHAGDRKRL EDWRIKGYDP LTFHOLLQHE FFIISAKGEEN
Ecoarog .....MNYON DDLRIKEIKE ULFFVALLLEK FFATENAANT
Aro4 MSESMPFAAN GMPKVNQGA E DVRILGYDP LASFALLQVQ IPATPTSLET
Ecoarof .....MQKDAL NNVHITDEQV LMTFEQLKAA FFLSLOQEAQ
Ecoaroh .....MNRT DELRTARIES LVTEAELALR YVTPGVATH

51                                     100
Aro3 IIAKADSVCD IILNGKDDRLV IVIGPCSLHD PKAAYDYADR LAKISEKLSK
Ecoarog VAHAKAIAHK IILKGNDDRL VVIGPCSLHD PVAAKEYATR LLALREFLKD
Aro4 AKRGFREAID IITEKDDRLV VVIGPCSLHD LEAAQEYALR LKKLSDELKG
Ecoarof IADSKXSISD IIAKDDPRL VVCGPCSLHD PETALEYARR FKALAAEVSD
Ecoaroh VTDSFRRIEK IILNGEDKRL VIIIGPCSLHD LTAAMEYATR LQSLRNQVGS

101                                    150
Aro3 DULIIMRAYL EKPRITVGNK GLINPDMNN SFQINKGLRI SFEMFIKLV
Ecoarog ELKIVMRVYF EKPRITVGNK GLINPDMN SFQINDGLRI ARKLLLDIND
Aro4 DLISIIMRAYL EKPRITVGNK GLINPDMNN TFNINKGLQS ARQLFVNLTN
Ecoarof SLYLVMRYF EKPRITVGNK GLINPDMG SFDVEAGLDI ARKLLLELVN
Ecoaroh RLEIVMRITF EKPRITVGNK GLINPDLNG SYRVNGGLGL ARKLLLVQNE

151                                    200
Aro3 EKLP IAGEML DTISQFLSD CFSLGAIGAR TTESQLHREL ASGLSFFVIGF
Ecoarog SGLPAAGEFL DMITPQYLAD LMSWGAIGAR TTESQVHREL ASGLSCFVGF
Aro4 IGLPIGSEML DTISQYLAD LVSFGAIGAR TTESQLHREL ASGLSFFVGF
Ecoarof MGLPLATEAL DPNSPQYLGD LFSWSAIGAR TTESQTHREM ASGLSFFVGF
Ecoaroh LGVITATEFL DNVTGRIAD LISWGAIGAR TTESQIHREM ASALSFFVGF

201                                    250
Aro3 KNGTGGGLQV AIDAMRAAH EHYLSVTKP GYTAIVGTGK NKOTFLILRG
Ecoarog KNGTGTIKV AIDAINAAGA PHCFLSVTKW GHSIAVNTSG NGDCHIILRG
Aro4 KNGT...LNV AVDACQAAAH SHFMGVTKH GVAAITTTKG NEHCFVILRG
Ecoarof KNGTGGSLAT AINAMRAAQ PHRFVGINQA GOVALLQDQ NPDGHVILRG
Ecoaroh KNGTDGNTRI AVDAIRAAQ HMF LSPDKN GOMTIYQTSG NPYGHILRG

251                                    300
Aro3 GKNGTNDKE SVONTKKQLE KAGLTDDSQK RIMIDDSHGN SNKDFKNOPK
Ecoarog GK EPNYSK HVAEVKEGLN KAGLP...A QVMIDFSHAN SSKDFKKOMD
Aro4 GKNGTNYDAK SVAEAKAQLP .AGS...N GLMIDYSHGN SNKDFRNOPK
Ecoarof GK APNYSKA DVAQCEKEME QAGLR...P SLMVDSSHGN SNKDYRROPA
Ecoaroh GKK.PNYHAD DIAAACDTLH EFDLP...E HLVVDSSHGN COKQHRROLE

301                                    350
Aro3 VAKCIYDOLT EGENSLOQVM IESNINEGND DIPKEGGREG LKYGCSITDA
Ecoarog VCADVCCQIA GGEKAIIGVM VESHLVEGND SL...E.SGEP LAYKCSITDA
Aro4 VNDVVCEQIA NGENAITQVM IESNINEGND GIPAE.GKAG LKYGVSITDA
Ecoarof VASVVAQIK DGNRSITGLM IESNINEGND S.SEQ.PRSE MKYGVSVTDA
Ecoaroh VCEDICQIR NGSTAIAGIM AESFLREGTD KIV...GSOP LTYGCSITOP

351                                    377
Aro3 CIGWESTEQV LELLAEGVRN RRKALKK
Ecoarog CIGWEDTDAL LROLANAVKA RRG....
Aro4 CIGWETTEDV LRKLAAVRO RREVNKK
Ecoarof CIGWEMTDAL LREIHQDLNG QLTARVA
Ecoaroh CIGWEDRQRL VEKLASAVDT RF.....

```

Figure 4.8 Sequence alignment of DAHP synthases from *S.cerevisiae* and *E.coli*

Sequences were aligned as described in section 4.6.3. Amino acid residues conserved in all five sequences are boxed. The sequence used to design the heterologous probe is hatched (Adapted from G. Braus, personal communication, 1991).

The rationale for the probe design as described in section 4.6.3 is illustrated. The amino acid sequence is shown with the corresponding codon favoured in *Streptomyces* sp. ; if two codons are equally favoured, a degeneracy is introduced in the third base position of the codon. In this case the sense strand sequence was used.

5' GAG AAG CCG CCG ACC ACC GTC GGC TGG AAG GGC CTC ATC AAC GGC CC 3'

C C G G G G G

sense strand

4.7.1 Comparison of the *S.coelicolor* DAHP synthase *N*-terminal sequence with the protein sequence data base

Among enzyme sequences bovine catalase showed the greatest similarity to the *S.coelicolor* DAHP synthase *N*-terminal sequence (Figure 4.10). A similarity rating (which scores positively for identical residues and for chemically similar residues and negatively for gaps introduced to improve alignment) of 12 was calculated for this comparison. One other protein, black beetle virus coat protein precursor, also scored 12, and the next highest score, 11, was human catalase. Four other protein sequences shared 7 or more identities with the 26 amino acid sequence of the *N*-terminus, without the introduction of any gaps in the sequences; these were *B.thuringiensis* parasporal crystal protein, *Rhodospirillum rubrum* ATP synthase, human cytochrome P450 (*Aro1*) and human apolipoprotein A precursor, respectively. Twenty-five proteins gave a similarity ratings of 10.

4.7.2 Comparison of the *S.coelicolor* DAHP synthase internal peptide sequence with the protein sequence data base

The unambiguously identified 12 residues of the common tryptic/clostripain peptide were compared with the protein sequence data base as above. Human mitochondrial NADH ubiquinone reductase with seven out of twelve residues matching shared the greatest similarity (Figure 4.11). Another thirty-two protein sequences possessed six out of twelve identities including members of the catalase family (Figure 4.10) and the *E.coli* phenylalanine sensitive DAHP synthase (Figure 4.12).

Figure 4.10 **Alignment of the *S.coelicolor* DAHP synthase sequence with bovine catalase**

Sequences were aligned as described in sections 4.7.1 and 4.7.2. Bovine catalase is compared with the first 26 amino acid residues of the *S.coelicolor* DAHP synthase and the clostripain/tryptic peptide sequence. Identical amino acids are indicated by double dots.

| | | | |
|--|---------------|----|---------------------|
| 17 | | 59 | |
| QRAAQKPDVLTTGGGNPVGDKLNSLTVGPRGPLLVDVVF | TDE | | bovine catalase |
| ::: ::: : :: : ::: : : : ::: : | | | |
| PENNQKP..LTTVAGAPVPDNQNSLTSG | ?PMLLQDVWFLEK | | <i>S.coelicolor</i> |
| 1 | 26 | 1 | 13 DAHP synthase |

Figure 4.11 **Alignment of the *S.coelicolor* DAHP synthase peptide sequence with NADH ubiquinone reductase**

Sequences were aligned as described in section 4.7.2 . NADH ubiquinone reductase is compared with the amino acid residues of the *S.coelicolor* DAHP synthase clostripain/tryptic peptide sequence. Identical amino acids are indicated by double dots.

| | | |
|---------------|-----|-----------------------------------|
| 549 | 560 | |
| PLLLLDLTWLEK | | NADH ubiquinone reductase |
| : : : : : | | |
| ?PMLLQDVWFLEK | | <i>S.coelicolor</i> DAHP synthase |

**Figure 4.12 Alignment of the *S.coelicolor* DAHP synthase with the
E.coli phenylalanine sensitive isozyme**

Sequences were aligned as described in section 4.7.2. *E.coli* DAHP synthase (phenylalanine inhibitable) is compared with the amino acid residues of the *S.coelicolor* DAHP synthase clostripain/tryptic peptide sequence. Identical amino acids are indicated by double dots, residues conserved between the three *E.coli* isozymes are asterixed.

| | | | | | |
|---------------|---|---|---|----|-----------------------------------|
| 15 | * | * | * | 26 | |
| KELLPPVALLEK | | | | | <i>E.coli aroG</i> |
| :: : :: | | | | | |
| ?PMLLQDVWFLEK | | | | | <i>S.coelicolor</i> DAHP synthase |

The first twenty-six amino acid residues of the *S.coelicolor* DAHP synthase were identified unambiguously from the *N*-terminus (Figure 4.1) and the sequence manually compared with the *N*-terminal regions of the *E.coli* isozymes (Schultz and Herrman, 1981), the phenylalanine sensitive isozyme from *S.cerevisiae* (Paravicini *et al.*, 1988) and the tryptophan activated enzyme from *Solanum tuberosum* (Dyer *et al.*, 1990). No significant identity was found although this was not unexpected as the first 41 residues of the otherwise highly homologous *E.coli* isozymes share only 6 amino acid identities (Ray *et al.*, 1988). Indeed, one must beware of drawing conclusions from *N*-terminal sequence data alone. Thus on the basis of the sequence of the first 40 amino acids of the *E.coli* isozymes it was wrongly concluded that the three isoenzymes evolved independently and not from a common ancestor (Poling *et al.*, 1981). However, when the complete sequences of the isozymes were determined (Davies and Davidson, 1982 ; Schultz *et al.*, 1984 ; Ray *et al.*, 1988) it became apparent that the three genes were highly homologous, with 40% of the amino acid sequence identical (Ray *et al.*, 1988).

Comparison of the *N*-terminal sequence with the protein sequence data base produced a potentially curious result. Bovine catalase, and to a lesser extent human and yeast catalases, exhibited the greatest similarity to the *S.coelicolor* DAHP synthase. It is hard to assess the significance of these findings. Assessing the significance of alignments over short regions (20-40 residues) presents problems if the alignment occurs with repeating amino acids or amongst the most frequently occurring amino acids (e.g. glycine, alanine, leucine). The chance of a match occurring by chance is significant (Doolittle, 1981) and the catalase alignment involves common amino acids. Because of these difficulties we do not regard these matches as significant and certainly do not regard them as evidence for any evolutionary relationship between catalase and DAHP synthase.

A further possibility is that the apparently conserved sequence is associated with a specific type of secondary structure. The corresponding region of the bovine catalase sequence lies between two α -helices of the *N*-terminal domain (Murthy *et al.*, 1981) and forms three β -turns. The *N*-terminal domain is involved in subunit interaction, is distinct from the catalytic and heme binding region of the enzyme, and is amongst the least conserved between the catalase sequences. The conserved *S.coelicolor* Gly-13 and Asn-20 residues (Figure 4.10) could similarly participate in the formation of two β -turns corresponding to two of the three β -turns found in the bovine catalase structure (Milner-White and Poet, 1987).

The region of greatest similarity between the *E.coli* isozymes is the stretch between residues 52 and 190, which includes blocks of virtually identical sequence (Ray *et al.*, 1988). It was against one of these sequence blocks that our conserved heterologous probe was designed.

We carried out proteolytic digests of intact *S.coelicolor* DAHP synthase in order to obtain enough suitable sequence with which to design oligonucleotide probes and to undertake comparative studies, with the hope of identifying a region sharing sequence identities with the published DAHP synthase sequences. Considering the amount of time spent preparing and purifying the peptides, we were disappointed with the quantity of sequence data we obtained. Only one peptide sequence was sufficiently long to be useful for probe design and sequence comparison analysis. WordSearch analysis yielded a set of proteins with six and, in one case, seven matches (Figure 4.11) with the twelve residues of the peptide sequence. One of these sequences was a DAHP synthase sequence, that of the *E.coli* phenylalanine-sensitive isozyme, but of the six identities found (Figure 4.12), only one was conserved in all three of the *E.coli* DAHP synthases. The sequence is located at the *N*-terminal region of the enzyme, and as this region is the least conserved between the

isozymes it seems unlikely that this sequence alignment is of any significance.

Therefore, we can conclude that this peptide is not derived from a region of the *S.coelicolor* DAHP synthase exhibiting strong sequence identity with a conserved region of the *E.coli* isozymes and the other protein alignments derived, including the best one, that with NADH ubiquinone reductase, would not appear to be significant.

The GC codon bias of streptomycetes, believed to have developed as a mechanism to enable control of cellular differentiation (Chater, 1989) may impose constraints on the amino acid composition of streptomycete proteins. The amino acid composition of globular proteins functioning in solution in an aqueous environment is remarkably similar (Creighton, 1983). However, residues such as Lys, Arg, Ile, whose favoured streptomycete codon possesses only one G or C base per codon triplet may be predicted to be less abundant than in organisms without such a codon bias. From our limited data, Lys and Ile with a frequency of 4.2 and 3.3% respectively, are significantly lower than the average values listed in the Atlas of Protein Sequence and Structure of 6 and 5%, respectively. Nevertheless, an overall comparison of many more streptomycete proteins requires to be undertaken before the generality of this conclusion can be confirmed.

In conclusion, the limited sequence data obtained from the *S.coelicolor* DAHP synthase is dissimilar to the homologous *E.coli* and *S.cerevisiae* isoenzymes. Overall sequence identity between the potato (Dyer *et al.*, 1990) and the other published sequences is less than 25%, and to detect this level of similarity a complete amino acid sequence was required, as some regions exhibited no similarity at all. A full comparison must therefore wait until more of the *S.coelicolor* sequence has been obtained.

Chapter 5 The cloning of the DAHP synthase gene
from *Streptomyces coelicolor*

5.1 Introduction

This chapter describes attempts to clone the *S.coelicolor* DAHP synthase gene by "reverse genetics". Hybridisation signals were identified following screening of genomic digests of *S.coelicolor* DNA with oligonucleotide probes. An appropriate restriction fragment was partially purified by size fractionation and used to generate an *S.coelicolor* genomic sub-library containing *Bam*H I fragments from 8-14 kb in size subcloned into vector pUC18. A genuine positive could not be isolated from this sub-library. A *S.coelicolor* genomic library constructed in λ GEM-11 was then screened. Two putative positive plaques were identified, isolated and purified. DNA was prepared from one isolate and analysed further. The pattern of hybridising restriction fragments was identical to that already seen for the genomic digests. It would appear likely that this phage insert contains the gene which codes for the *S.coelicolor* DAHP synthase.

5.2 The oligonucleotides used as probes

Amino acid sequence derived from the *S.coelicolor* DAHP synthase was used to design two homologous oligonucleotide probes using the rationale outlined in section 4.6. A third heterologous oligonucleotide designed against a sequence conserved between the *E.coli* and *S.cerevisiae* DAHP synthase isoenzymes (Figure 4.8) and implicated in catalysis (Ray *et al.*, 1988) was also synthesised (section 4.6.3). These oligonucleotides designed to detect the coding sequences of the *N*-terminal region, the internal peptide, and the putative active site peptide, respectively, are shown (Figure 4.6, 4.7 and 4.9).

5.3 Southern blot analysis of digests of *S.coelicolor* genomic DNA

S.coelicolor genomic DNA was prepared and digested with a range of restriction enzymes as described in Materials and Methods (sections 2.9, 2.12). 5 μ g of genomic DNA was

used for each digest. 10 µg of total mammalian DNA (haploid genome size of 3×10^9 bp) is required to detect sequences which occur at the single copy level on a Southern blot (Sambrook *et al.*, 1989). It was assumed that the *S.coelicolor* DAHP synthase gene would be present in a single copy within the *S.coelicolor* genome (genome size 8×10^6 bp) (D. Hopwood, personal communication, 1991). Therefore, a digest of 5 µg of genomic DNA was estimated to be sufficient to allow detection.

The cleaved DNA was separated by agarose gel electrophoresis as described in section 2.13 (Figure 5.1). To improve the resolution of the separated DNA bands, gels were run overnight at 20 V using a Tris-acetate buffer system. The DNA was then transferred to nylon filters as described in section 2.17, and the filters probed with the appropriate oligonucleotides following the method of Binnie, 1990.

5.3.1 Probing with the *N*-terminal oligonucleotide

Filters containing genomic *S.coelicolor* DNA cut with one of four restriction enzymes were prepared (section 5.3) and prehybridised as described in section 2.19.

The *N*-terminal oligonucleotide was end-labelled with ^{32}P and separated from unincorporated label as described in section 2.18. Aliquots of the radioactively labelled oligonucleotide were then added to a polythene bag containing the filter and the hybridisation solution. The hybridisation solution was as follows:- 6 x SSC, 0.05% (w/v) sodium pyrophosphate, 0.05% (w/v) SDS. The bag was sealed and probe plus filter incubated as described in section 2.19. Hybridisation reactions were carried out at a range of temperatures, 60°C being the highest temperature employed.

The hybridised filters were then washed as described in section 2.19. The oligonucleotide remained bound when washed at a temperature of 65°C and ionic strength of 1 x SSC.

Filters were then autoradiographed as described in section 2.19 for 38 hours. Autorads

from a lower stringency hybridisation (hybridising at 50°C, washing at 55°C) (Figure 5.2) and a higher stringency (hybridising at 60°C, washing at 65°C) (Figure 5.3) are also shown.

At the highest stringency (hybridising at 60°C in 6 x SSC ; washing at 65°C in 1 x SSC), a single hybridising restriction band is seen for each digest. The size of the hybridising DNA band for each restriction digest following incubation with the *N*-terminal probe is given in Table 5.1.

5.3.2 Probing with the peptide oligonucleotide

Fresh filters were prepared as before (section 5.3) or filters re-used following the removal of old probe (section 2.23). The probe labelling and hybridisation reactions were carried out as before (section 5.3.1). Hybridisation reactions were carried out at a range of temperatures, 60°C being the highest temperature employed. The oligonucleotide remained bound when washed at a temperature of 65°C and ionic strength of 1 x SSC.

At the highest stringency (hybridising at 60°C in 6 x SSC ; washing at 65°C in 1 x SSC) a single hybridising restriction band is seen following autoradiography for 40 hours (Figure 5.4). The size of the hybridising DNA band for each restriction digest following incubation with the peptide probe is given (Table 5.1). In this case the *Pst* I digest track contains no signal. On closer analysis it became apparent that one of the possible degenerate sequences corresponding to our probe contains a *Pst* I restriction site. The DNA must have been cleaved in this region preventing hybrid formation.

5.3.3 Probing with the consensus oligonucleotide

Fresh filters were prepared as before (section 5.3) or filters re-used following the removal of old probe (section 2.23). The probe labelling and hybridisation reactions were carried

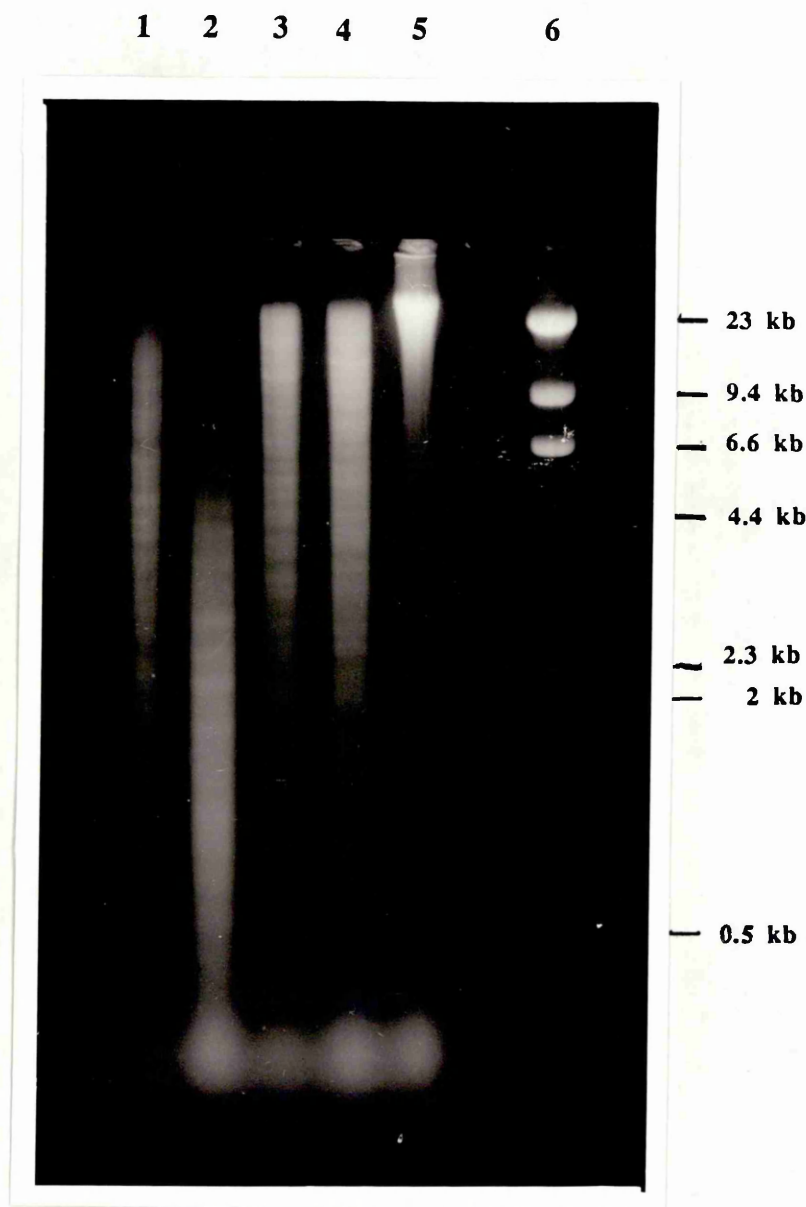


Figure 5.1 Agarose gel electrophoresis of restriction digests of *S.coelicolor* genomic DNA

Gel was run as described in section 5.3. 5 µg of DNA per lane was digested (section 2.12) with the following the enzymes :-

Lane 1 : *Bam*H I

Lane 2 : *Sal* I

Lane 3 : *Sst* I

Lane 4 : *Pst* I

Lane 5 : uncut genomic DNA

Lane 6 : λ -*Hind* III M_r markers

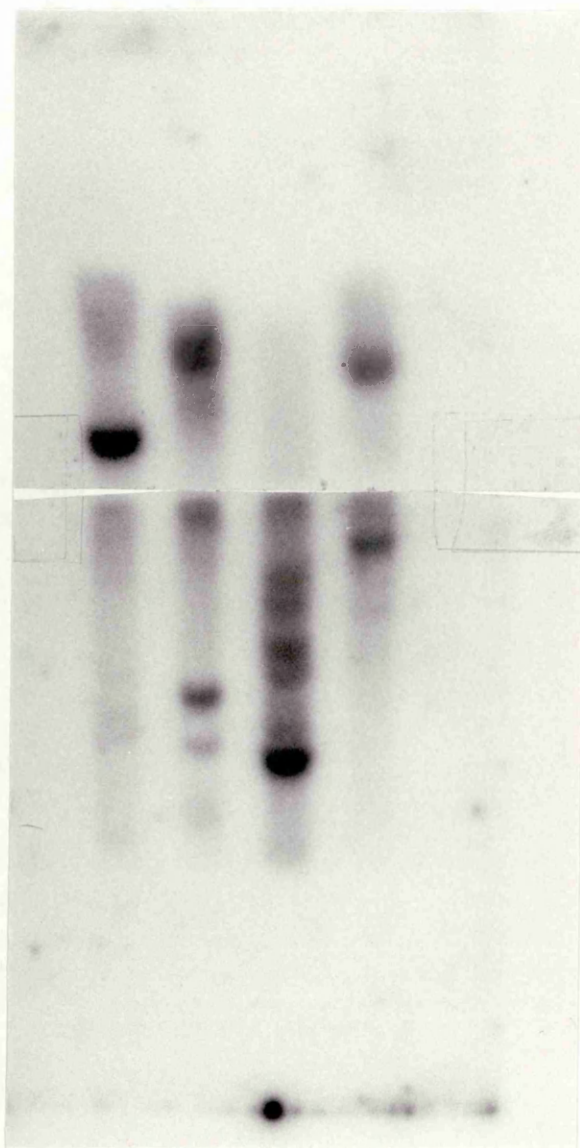


Figure 5.2 **Autoradiograph of a Southern blot of digested genomic *S.coelicolor* DNA probed with the *N*-terminal oligonucleotide**

DNA was digested with a range of restriction enzymes, the products separated by agarose electrophoresis (Figure 5.1) and transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the *N*-terminal oligonucleotide was carried out under less stringent hybridisation (50°C, 6 x SSC) and washing (55°C, 6 x SSC) conditions as described in section 5.3.1.

Lane 1 : *Sst* I

Lane 2 : *Bam*H I

Lane 3 : *Sal* I

Lane 4 : *Pst* I

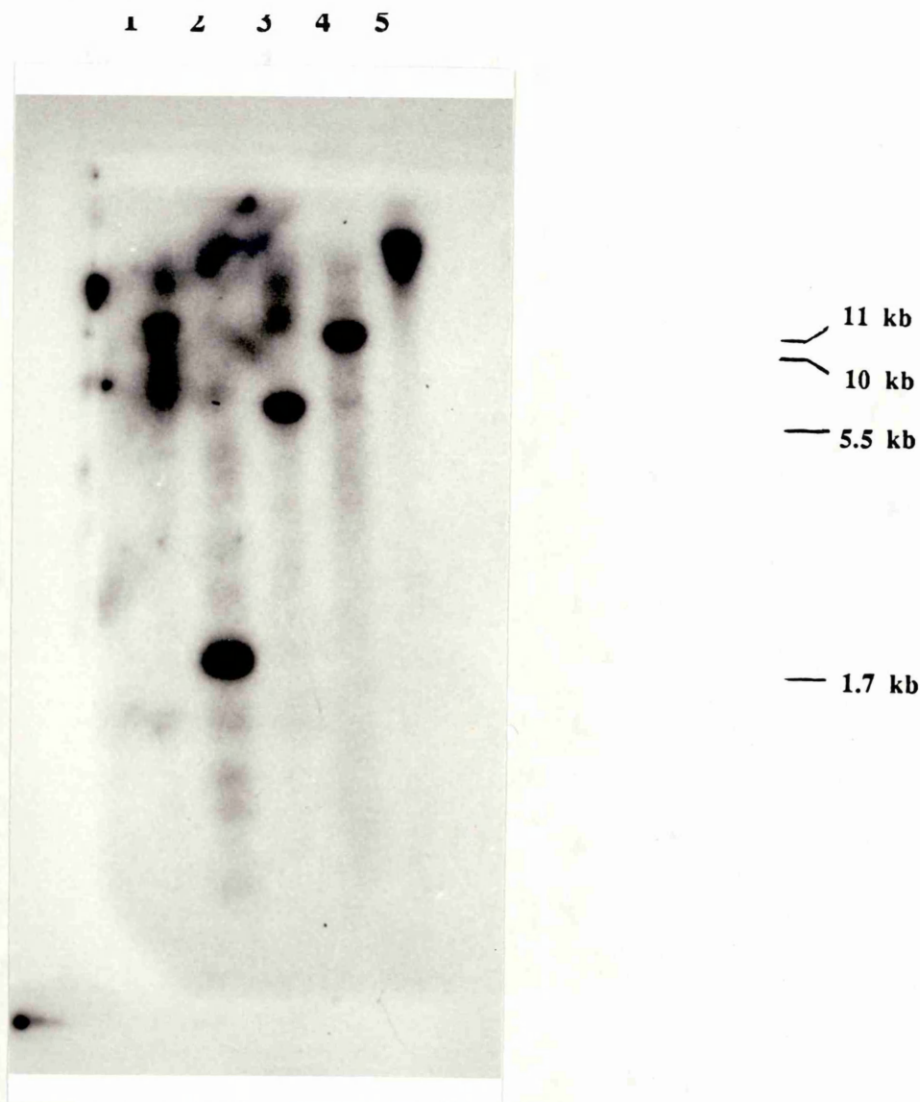


Figure 5.3 **Autoradiograph of a Southern blot of digested genomic *S.coelicolor* DNA probed with the *N*-terminal oligonucleotide**

DNA was digested with a range of restriction enzymes, the products separated by agarose electrophoresis (Figure 5.1) and transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the *N*-terminal oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 1 x SSC) conditions as described in section 5.3.1.

Lane 1 : *Bam*H I

Lane 2 : *Sal* I

Lane 3 : *Sst* I

Lane 4 : *Pst* I

Lane 5 : uncut genomic DNA

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.1).

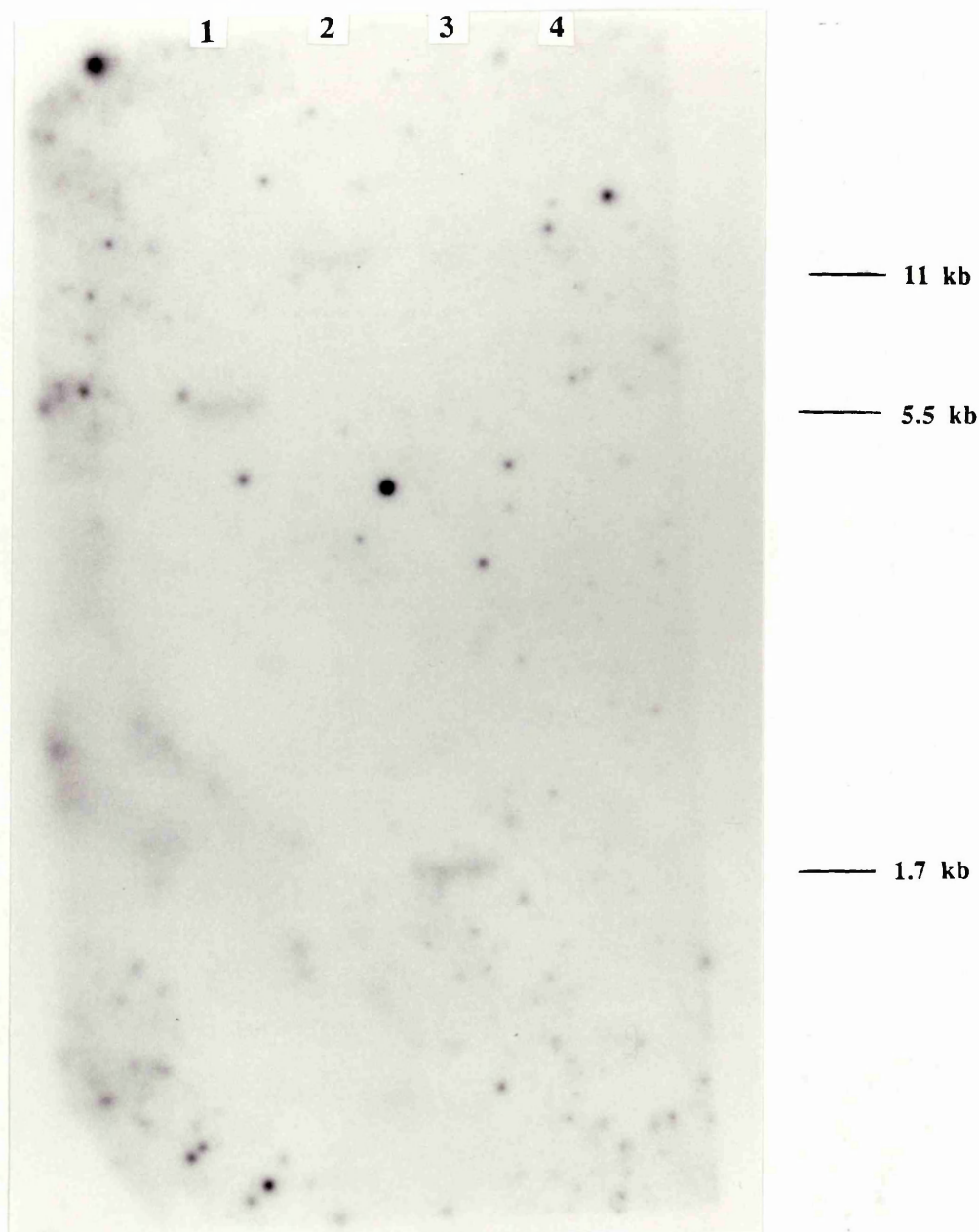


Figure 5.4 **Autoradiograph of a Southern blot of digested genomic *S.coelicolor* DNA probed with the peptide oligonucleotide**

DNA was digested with a range of restriction enzymes, the products separated by agarose electrophoresis (Figure 5.1) and transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the peptide oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 1 x SSC) conditions as described in section 5.3.2.

Lane 1 : *Sst* I

Lane 2 : *Bam*H I

Lane 3 : *Sal* I

Lane 4 : *Pst* I

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.1).

Table 5.1 **Size of hybridising restriction fragments obtained from digests of *S.coelicolor* genomic DNA**

| Restriction enzyme(s) | Probe | Fragment size(kb) | Reference |
|------------------------------|--------------------|-------------------|------------|
| <i>Sst</i> I | <i>N</i> -terminal | 5.5 | Figure 5.3 |
| " | peptide | " | Figure 5.4 |
| <i>Bam</i> H I | <i>N</i> -terminal | 11 | Figure 5.3 |
| " | peptide | " | Figure 5.4 |
| <i>Sal</i> I | <i>N</i> -terminal | 1.7 | Figure 5.3 |
| " | peptide | " | Figure 5.4 |
| <i>Pst</i> I | <i>N</i> -terminal | 10 | Figure 5.3 |
| " | peptide | none | Figure 5.4 |
| <i>Sph</i> I | <i>N</i> -terminal | 5.6 | Figure 5.6 |
| " | peptide | " | Figure 5.7 |
| <i>Kpn</i> I | <i>N</i> -terminal | 8 | Figure 5.6 |
| " | peptide | " | Figure 5.7 |
| <i>Bam</i> H I/ <i>Pst</i> I | <i>N</i> -terminal | 5.9 | Figure 5.6 |
| " | peptide | none | Figure 5.7 |
| <i>Pst</i> I/ <i>Sst</i> I | <i>N</i> -terminal | 4.7 | Figure 5.6 |
| " | peptide | none | Figure 5.7 |
| <i>Pst</i> I/ <i>Kpn</i> I | <i>N</i> -terminal | 4.5 | Figure 5.6 |
| " | peptide | none | Figure 5.7 |

out as before (section 5.3.1). Hybridisation reactions were carried out at a range of temperatures between 50°C and 60°C and the filters were washed in high salt buffer (6 x SSC) within the same temperature range.

Following autoradiography (40 hour exposure) no discrete hybridising restriction fragments could be visualised. At the lowest stringency (hybridising and washing at 50°C in 6 x SSC), a smear was present in the area of the high M_r DNA on the autorad. This would appear to be the result of non-specific hybridisation. At moderately more stringent conditions (hybridising at 50°C and washing at 55°C in 6 x SSC) this high M_r signal was removed and no other bands were seen.

Consequently, use of this probe was discontinued.

5.4 Choosing an appropriately sized restriction fragment to clone

In selecting an optimal restriction digest fragment for cloning the following criteria were employed :-

(1) the fragment must be large enough to ensure a reasonable chance of cloning the entire DAHP synthase gene as opposed to only part of the gene.

The subunit size of the *S.coelicolor* DAHP synthase would indicate a polypeptide composed of around 500 amino acids, which could be encoded by a DNA fragment of around 1.5 kb. Allowing for a degree of overlap either side of the coding region it was decided that a restriction fragment of around 5 kb would be ideal.

(2) the fragment must be generated by the action of restriction enzymes found in the plasmid pUC18 polylinker region.

This would allow us to use the pUC18 plasmid, one of a series, which in addition to carrying a selectable antibiotic marker, possess IPTG-inducible histochemical selection when used in an appropriate host (section 2.16.3). This property facilitates easy identification of recombinants.

(3) ideally the fragment should be generated by the action of two restriction enzymes found in the pUC18 polylinker region, thus allowing directional cloning of the DNA fragment and preventing self-ligation of the plasmid.

(4) if a double digest fragment of the appropriate size could not be obtained then a restriction enzyme cutting to leave a 5' phosphate overhang would be the next choice. Enzymes which leave a 3' hydroxyl overhang cannot be readily dephosphorylated by alkaline phosphatase at the 5' position and such overhanging linearised plasmids readily self-ligate.

5.4.1 Digestion of *S.coelicolor* genomic DNA with pairs of restriction enzymes

S.coelicolor genomic DNA was prepared and digested with a range of restriction enzymes whose recognition sequences lie within in the pUC18 polylinker cloning region. Single and double digests were carried out, cleaved DNA fragments separated by agarose electrophoresis (Figure 5.5) and filters prepared as before (section 5.3).

Filters were incubated with the *N*-terminal probe and washed under the conditions of highest stringency previously described (section 5.3.1). Filters were autoradiographed for 80 hours and a pattern of hybridising restriction fragments obtained (Figure 5.6). The size of each hybridising DNA band is given (Table 5.1).

Filters were stripped of probe (section 2.23) and the procedure repeated with the internal peptide oligonucleotide (Figure 5.7). The size of each hybridising DNA band is given

23 kb —
 9.4 kb —
 6.6 kb —
 4.4 kb —

 2.3 kb —
 2 kb —

 0.5 kb —

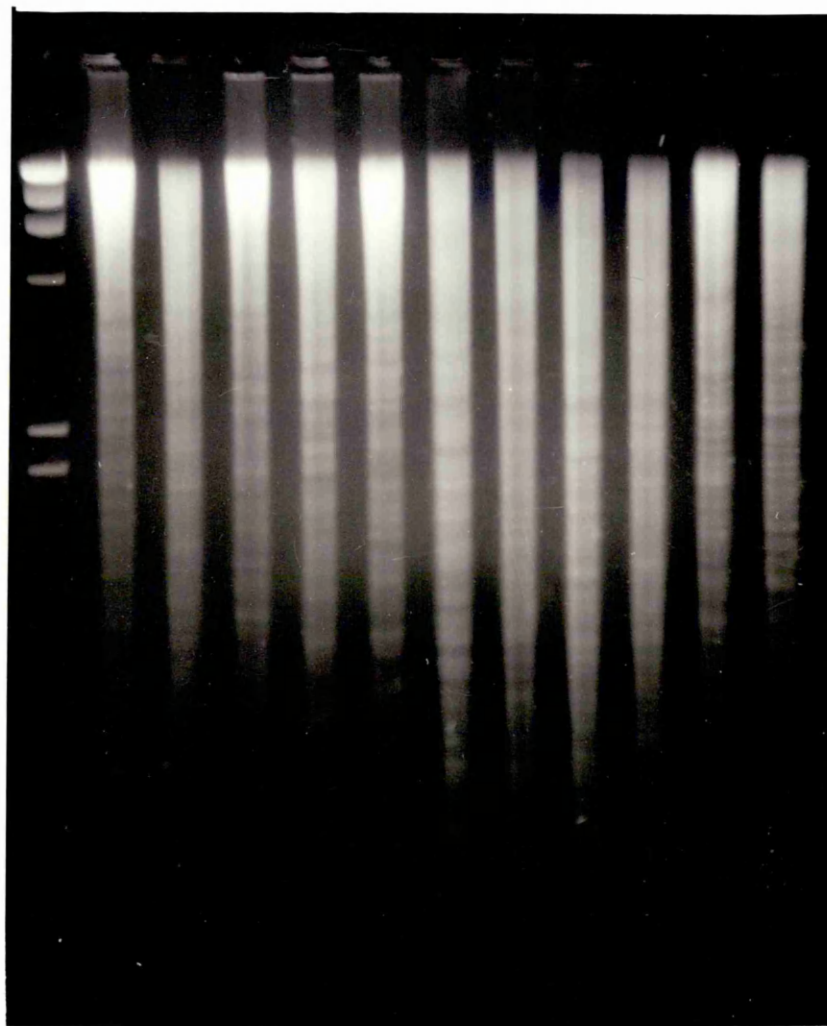


Figure 5.5 Agarose gel electrophoresis of double and single restriction digests of *S.coelicolor* genomic DNA

Gel was run as described in section 5.4.1. 5µg of DNA per lane was digested (section 2.12) with the following the enzymes :-

- Lane 1 : *λ-Hind* III M_r markers
- Lane 2 : *Sst* I
- Lane 3 : *Bam*H I
- Lane 4 : *Pst* I
- Lane 5 : *Sph* I
- Lane 6 : *Kpn* I
- Lane 7 : *Bam*H I / *Sst* I
- Lane 8 : *Bam*H I / *Pst* I
- Lane 9 : *Bam*H I / *Sph* I
- Lane 10 : *Bam*H I / *Kpn* I
- Lane 11 : *Pst* I / *Sst* I
- Lane 12 : *Pst* I / *Kpn* I

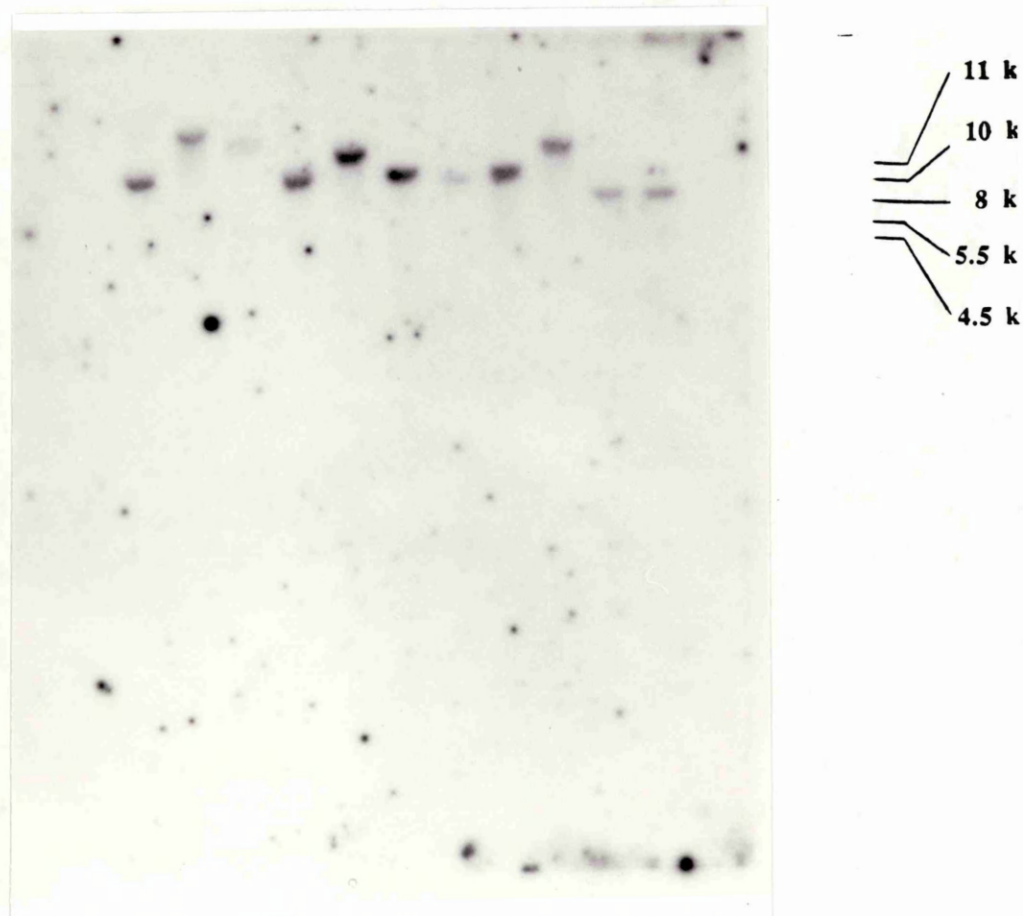


Figure 5.6 **Autoradiograph of a Southern blot of single and double digests of genomic *S.coelicolor* DNA probed with the *N*-terminal oligonucleotide**

DNA from a genomic digest (Figure 5.5) was transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the *N*-terminal oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 1 x SSC) conditions as described in section 5.3.1.

| | |
|-----------|-------------------------------|
| Lane 1 : | <i>Sst</i> I |
| Lane 2 : | <i>Bam</i> H I |
| Lane 3 : | <i>Pst</i> I |
| Lane 4 : | <i>Sph</i> I |
| Lane 5 : | <i>Kpn</i> I |
| Lane 6 : | <i>Bam</i> H I / <i>Sst</i> I |
| Lane 7 : | <i>Bam</i> H I / <i>Pst</i> I |
| Lane 8 : | <i>Bam</i> H I / <i>Sph</i> I |
| Lane 9 : | <i>Bam</i> H I / <i>Kpn</i> I |
| Lane 10 : | <i>Pst</i> I / <i>Sst</i> I |
| Lane 11 : | <i>Pst</i> I / <i>Kpn</i> I |

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.1). Where the product of a double digestion is not smaller than the single digestion product for either of the individual restriction enzymes (ie. the digests in Lanes 6, 8, 9, respectively) the fragment size is not listed.

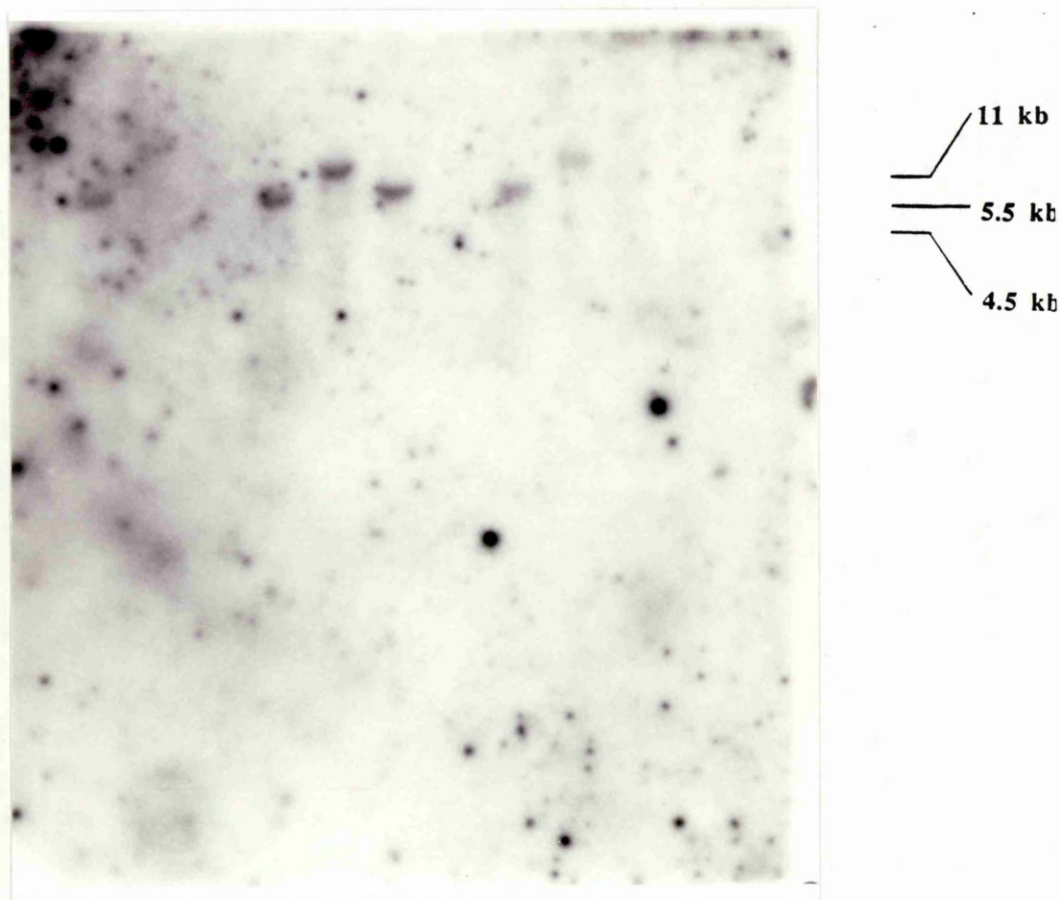


Figure 5.7 **Autoradiograph of a Southern blot of single and double digests of genomic *S.coelicolor* DNA probed with the peptide oligonucleotide**

DNA from a genomic digest (Figure 5.5) was transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the peptide oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 1 x SSC) conditions as described in section 5.3.2.

| | |
|-----------|-------------------------------|
| Lane 1 : | <i>Sst</i> I |
| Lane 2 : | <i>Bam</i> H I |
| Lane 3 : | <i>Pst</i> I |
| Lane 4 : | <i>Sph</i> I |
| Lane 5 : | <i>Kpn</i> I |
| Lane 6 : | <i>Bam</i> H I / <i>Sst</i> I |
| Lane 7 : | <i>Bam</i> H I / <i>Pst</i> I |
| Lane 8 : | <i>Bam</i> H I / <i>Sph</i> I |
| Lane 9 : | <i>Bam</i> H I / <i>Kpn</i> I |
| Lane 10 : | <i>Pst</i> I / <i>Sst</i> I |
| Lane 11 : | <i>Pst</i> I / <i>Kpn</i> I |

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.1). Where the product of a double digestion is not smaller than the single digestion product for either of the individual restriction enzymes (ie. the digests in Lanes 6, 8, 9, respectively) the fragment size is not listed.

(Table 5.1).

5.4.2 The choice of restriction fragment

The results obtained from probing double and single digests of *S.coelicolor* genomic DNA were used to decide which restriction fragment to clone. The GC bias of *Streptomyces* DNA now hindered our efforts as conventional restriction enzymes cut GC rich DNA relatively infrequently.

Double restriction digests employing polylinker restriction enzymes gave only two suitably sized 5 kb fragments, a *Pst* I/*Kpn* I fragment of 4.5 kb and a *Pst* I/*Sst* I fragment 4.7 kb (Table 5.1), but as the internal peptide probe contains a *Pst* I site they cannot be used as they could not contain the whole gene (section 5.3.2).

Digestion with the restriction enzymes *Sst* I and *Sph* I gave a suitably sized fragments of 5.5 kb and 5.6 kb, respectively (Table 5.1) but these enzymes cut to leave 3' hydroxyl overhangs and were not suitable (section 5.4).

The 1.7 kb fragment generated by the action of the *Sal* I enzyme was considered too small to have a reasonable chance of containing the whole gene (Table 5.1) (section 5.4)

A range of single restriction enzyme digests gave fragment sizes between 8 and 11 kb (Table 5.1) including those generated by *Kpn* I (8 kb), *Pst* I (10 kb) and *Bam*H I (11 kb). *Kpn* I cuts to leave a 3' hydroxyl overhang and the *Pst* I fragment is also not suitable (section 5.3.2, 5.4), therefore it was decided to isolate and enrich for the 11 kb *Bam*H I fragment and to ligate it into an appropriately cut and dephosphorylated preparation of plasmid pUC18. (This procedure was made easier with the availability of plasmid ready cut with *Bam*H I and treated with bacterial alkaline phosphatase.)

5.5 Attempts to isolate the *S.coelicolor* DAHP synthase gene from a genomic sub-library in pUC18

5.5.1 Size fractionation and purification of fragments from a *Bam*H I digestion of *S.coelicolor* genomic DNA

A sample of *S.coelicolor* genomic DNA was digested exhaustively with *Bam*H I (section 2.12) and the resulting fragments separated by agarose electrophoresis (section 2.13) (Figure 5.8). Restriction fragments of the appropriate size were located, excised from the gel, and recovered from the gel by electroelution (section 2.14.2). The sizes of the recovered fragments were estimated by agarose gel analysis. DNA from the genomic digest gel was blotted onto nylon filters by the method of Southern (section 2.17) and probed with the *N*-terminal oligonucleotide (section 5.3.1) to verify that the correct fragment size of DNA had been excised and purified from the gel.

5.5.2 Ligation of *Bam*H I fragments into plasmid pUC18

Purified *Bam*H I DNA fragments (section 5.5.1) were ligated into plasmid pUC18 as described in section 2.15. Appropriate test ligations were carried out using cut λ -DNA (section 2.1.5) and agarose gel analysis was carried out to establish that control and sample ligations had been successful.

5.5.3 Transformation of *E.coli* with plasmid DNA

Test plasmid (uncut pUC18) and plasmid ligated with the purified *Bam*H I fragments were used to transform *E.coli* as described in section 2.16. Following each round of transformation recombinants were picked and retained for screening. Plasmid DNA was made from a selection of recombinants (section 2.10) and digested with *Bam*H I to check that the size of the insert in the plasmids was correct.

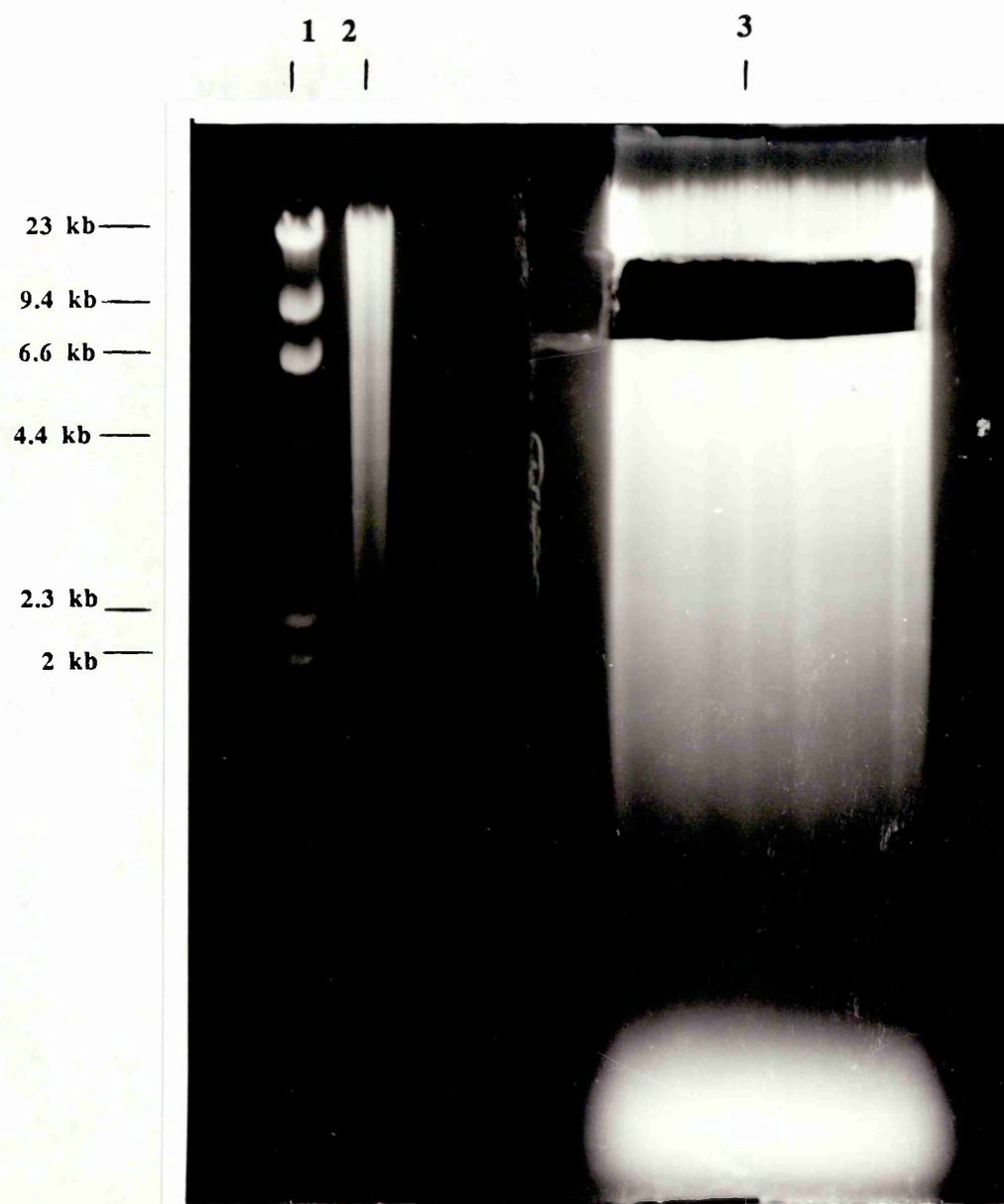


Figure 5.8 Purification and electroelution of *Bam*H I restriction fragments of 8-14 kb in size from a total digest of *S.coelicolor* genomic DNA

The gel was run and the *Bam*H I fragments electroeluted as described in section 5.5.1. 150 µg of DNA was digested (section 2.12) with the restriction enzyme *Bam*H I :-

Lane 1 : λ -*Hind* III M_r markers

Lane 2 : *Bam*H I digestion products ($1/30$ th of total digest)

Lane 3 : *Bam*H I digestion products

(the remainder of the digest with excised band removed)

5.5.4 Screening recombinants for the *S.coelicolor* DAHP synthase gene by colony hybridisation

Recombinant pUC clones were screened (570 in total) as described in section 2.20 with both the *N*-terminal and the internal peptide oligonucleotide probes. Positive clones could not be identified when hybridisation reactions were carried using the conditions employed for earlier hybridisation experiments involving *S.coelicolor* genomic DNA (section 5.3). This method of isolating the *S.coelicolor* DAHP synthase gene was therefore abandoned.

5.6 Attempts to isolate the *S.coelicolor* DAHP synthase gene from a genomic library constructed in λ GEM-11

A *S.coelicolor* genomic library was constructed in λ GEM-11 by Mr. R.D. Taylor (Departments of Biochemistry and Genetics, University of Glasgow). Total genomic DNA was partially digested with the frequently cutting restriction enzyme *Sau* 3A, generating a near random pattern of fragments. 11-14 kb size fragments were purified and ligated with the λ GEM-11 arms to generate the primary library of recombinant phage. This library was then amplified, stored and used as required in the gene screening programme.

5.6.1 Primary screening

Plating bacteria were prepared (section 2.11.1 (a)) and infected with phage of titre measured (section 2.11.1) to be 2000 plaque forming units per plate (section 2.21(a)). Plates were incubated overnight and impressions of the plate onto nylon filters obtained as described in section 2.21 (a).

The filters were prepared (section 2.20) and probed with the *N*-terminal oligonucleotide probe (sections 2.19, 5.3.1). Following autoradiography for 124 hours two putative positive signals were identified and designated λ GW 4 (Figure 5.9) and λ GW 5 (Figure

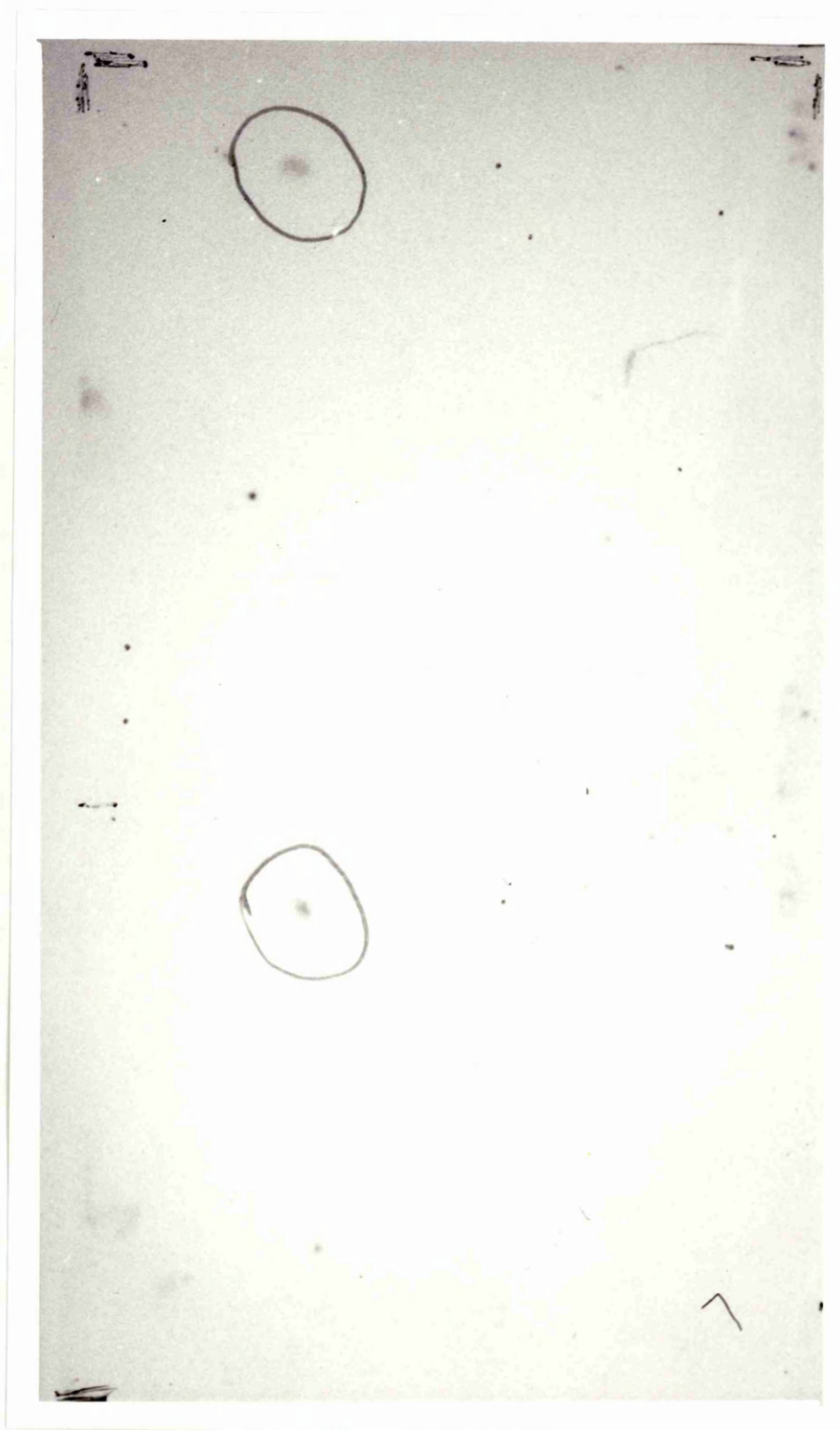


Figure 5.9 **Autoradiograph of primary filter 4 screened with the *N*-terminal oligonucleotide**

Filters were prepared and screened with the *N*-terminal oligonucleotide as described in section 5.6.1. The large, strongly hybridising plaque λ GW 4 (circled) was purified to homogeneity and further characterised. Other spots were spurious as they did not occur on a duplicate filter.

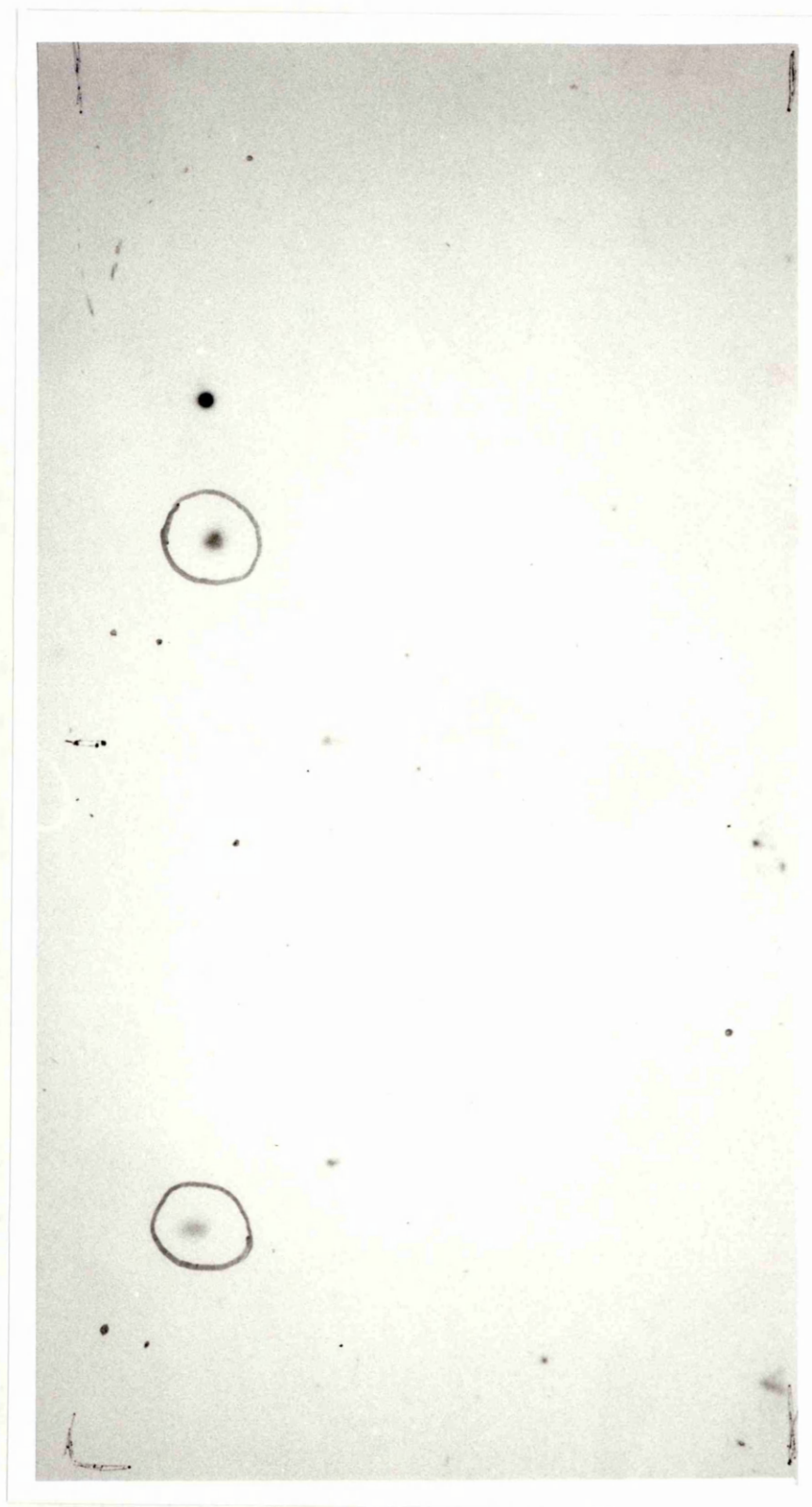


Figure 5.10 **Autoradiograph of primary filter 5 screened with the *N*-terminal oligonucleotide**

Filters were prepared and screened with the *N*-terminal oligonucleotide as described in section 5.6.1. The large, strongly hybridising plaque λGW 5 (circled) was purified to homogeneity and further characterised. Other spots were spurious as they did not occur on a duplicate filter.

5.10), respectively.

5.6.2 Secondary screening

The putative positive plaques were isolated (section 2.11.2) and purified (section 2.21 (b)). Secondary filters were prepared as before (sections 2.20, 2.21 (a)), with 15 and 12 plaques picked and streaked out for the λ GW 4 and the λ GW 5 isolates, respectively.

The filters were then probed with the *N*-terminal oligonucleotide as before (sections 2.19, 5.31). Following autoradiography for 13 hours the positive signals were confirmed. 2 out of 15 of the plaques purified as the λ GW 4 isolate were positive (Figure 5.11), and 1 out of the 12 plaques purified as the λ GW 5 isolate was positive (Figure 5.12).

5.6.3 Characterisation of the hybridising plaque λ GW 4

DNA was prepared from the λ GW 4 isolate (section 2.11.4), digested with *Sal* I, *Bam*HI and *Sst* I (section 2.12) and the digestion products separated by agarose gel electrophoresis (section 2.13) (Figure 5.13). Two bands corresponding to the λ GEM-11 arms were present in each of the lanes containing recombinant phage together with one or more genomic DNA insert bands.

The Southern blot of this gel (section 2.17) was probed with both the *N*-terminal and internal peptide oligonucleotides as before (sections 2.19, 5.3.1, 5.3.2). Following autoradiography, a single hybridising band is seen for each digest (Figures 5.14, 15). The size of these bands (Table 5.2) corresponds to those obtained from analysis of genomic digests of *S.coelicolor* DNA (Table 5.1). This confirms that the homology to the probe lies within the insert bands and establishes the authenticity of the clone.

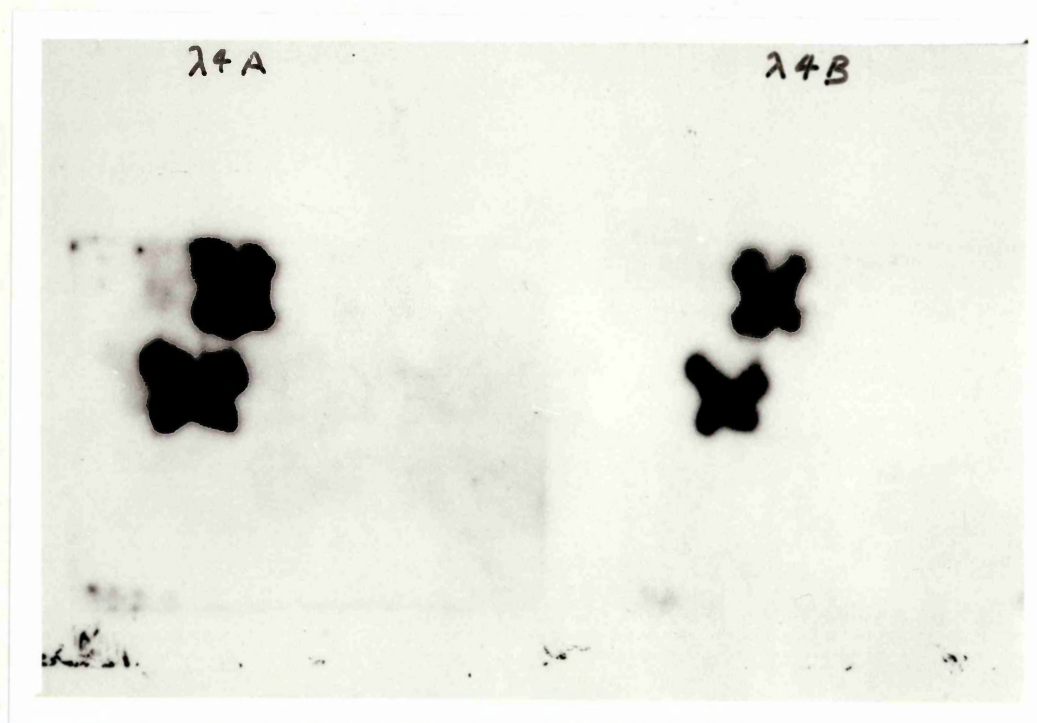


Figure 5.11 **Autoradiograph of the λ GW 4 secondary filter screened with the *N*-terminal probe**

The putative positive plaque λ GW 4 was isolated, purified and duplicate secondary filters prepared as described in section 5.6.2. Positive plaques were indicated by dark crosses and plugs were prepared from these for long-term storage (section 2.11.2).

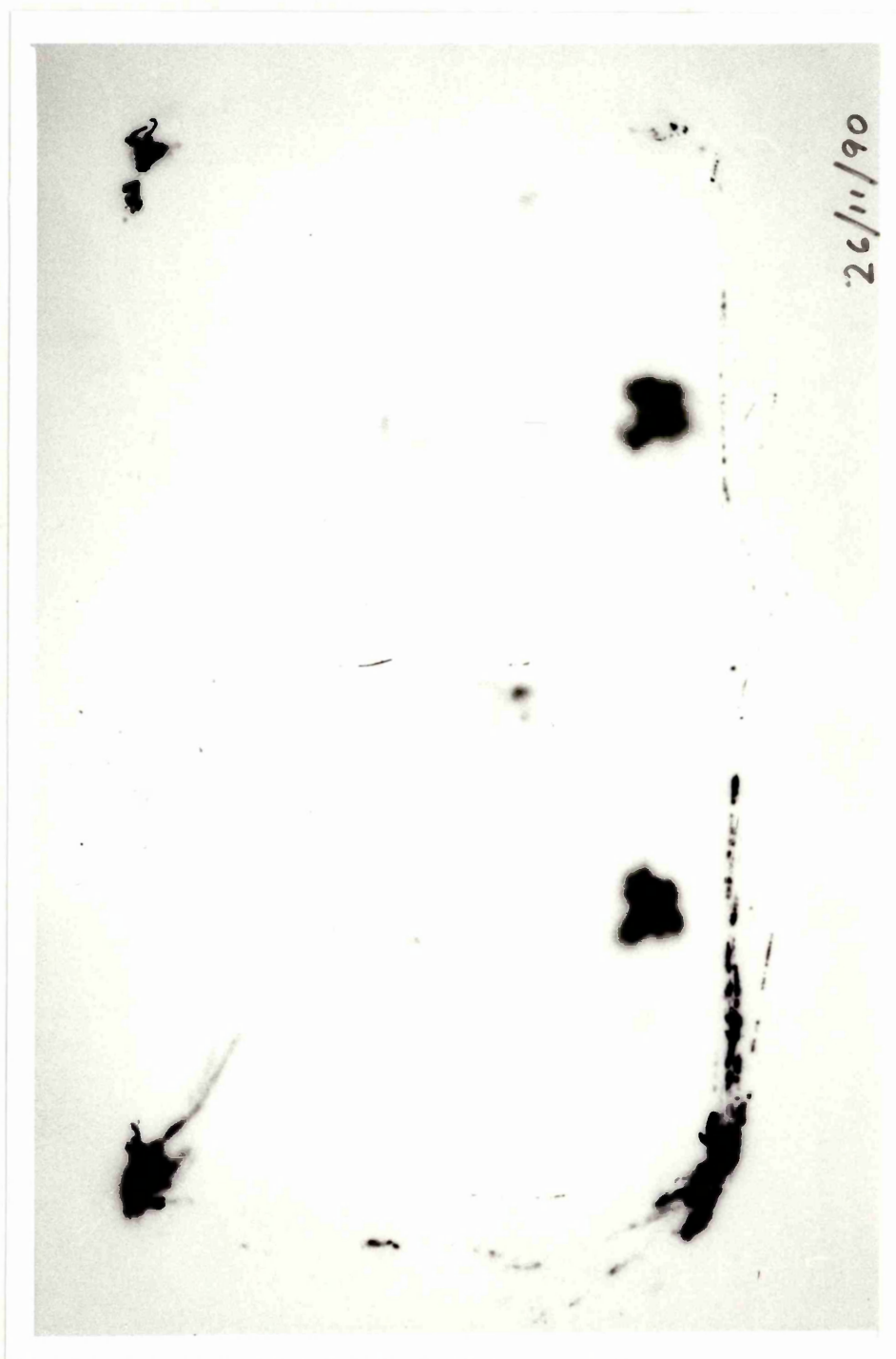


Figure 5.12 **Autoradiograph of the λ GW 5 secondary filter screened with the N-terminal oligonucleotide**

The putative positive plaque λ GW 5 was isolated, purified and duplicate secondary filters prepared as described in section 5.6.2. The positive plaque was indicated by a dark cross and plugs were prepared from this for long-term storage (section 2.11.2).

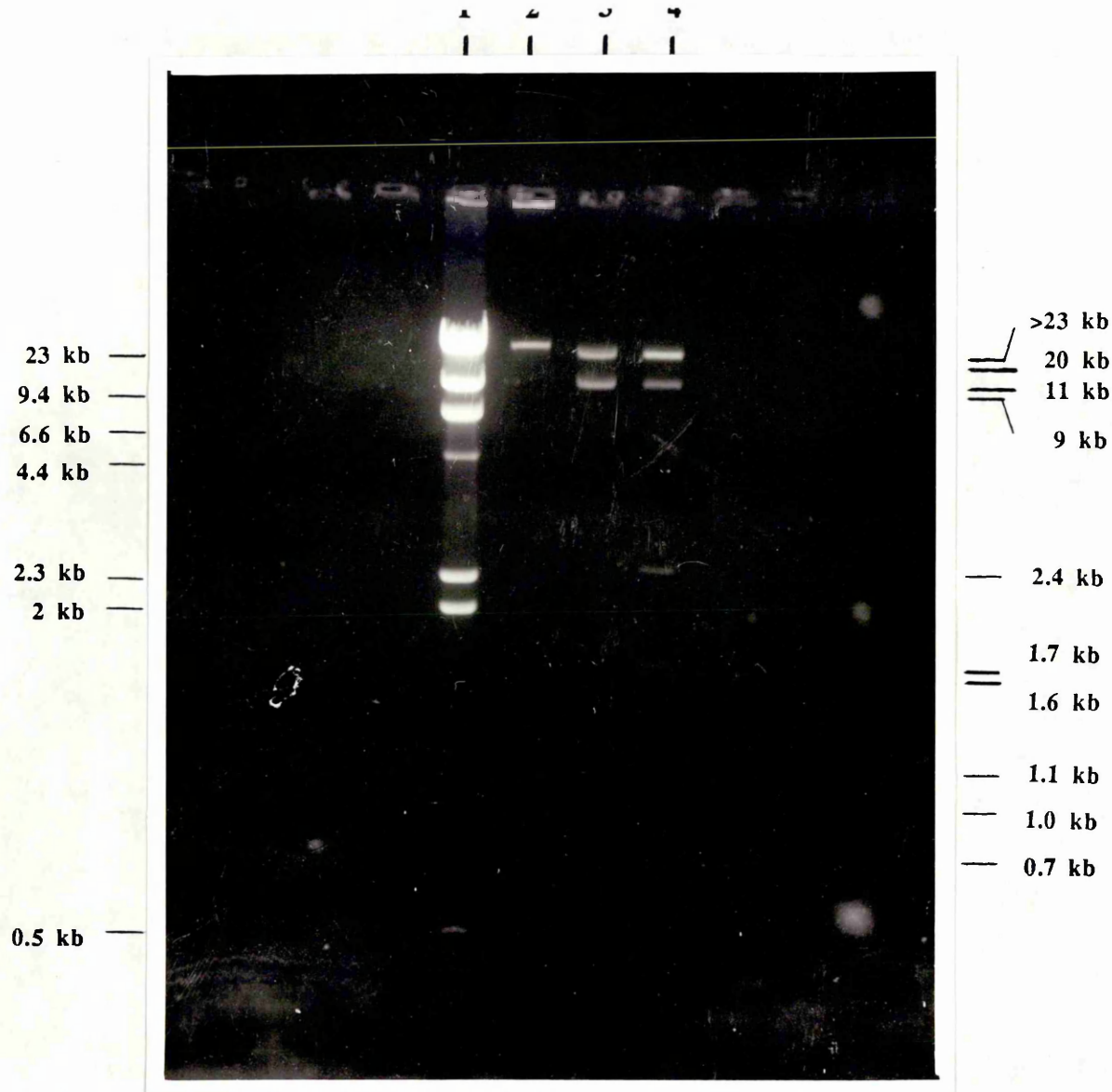


Figure 5.13 Agarose gel electrophoresis of restriction digests of λGW 4 DNA

DNA was prepared from λGW 4 as described in section 5.6.3. 0.5 µg of λGW 4 DNA per lane was digested (section 5.6.3) with the following the enzymes :-

- Lane 1 : λ-*Hind* III M_r markers
- Lane 2 : uncut
- Lane 3 : *Bam*H I
- Lane 4 : *Sal* I

Each digest possesses two bands of 20 kb and 9 kb which correspond to the size of the arms of the λGEM-11 vector. Lane 2 possesses a single band of >23 kb which represents uncut λGW 4. For the *Bam*H I and *Sal* I digests the insert fragment sizes are listed above. An *Sst* I digest was also prepared and product fragments separated by agarose electrophoresis (data not shown). A fragment of 5.5 kb was identified, but smaller fragments could not be visualised.

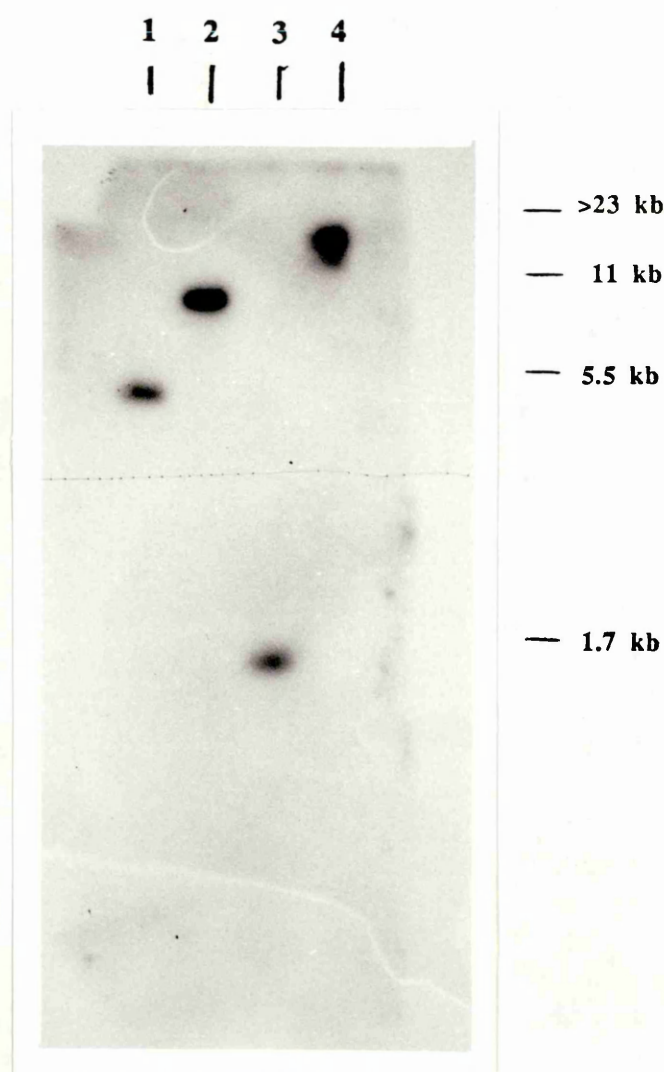


Figure 5.14 **Autoradiograph of a Southern blot of digested λ GW 4 DNA probed with the *N*-terminal oligonucleotide**

λ GW 4 DNA was digested with a range of restriction enzymes (Figure 5.11) and was transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the *N*-terminal oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 6 x SSC) conditions as described in section 5.6.3.

Lane 1 : *Sst* I
 Lane 2 : *Bam*H I
 Lane 3 : *Sal* I
 Lane 4 : uncut

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.2).

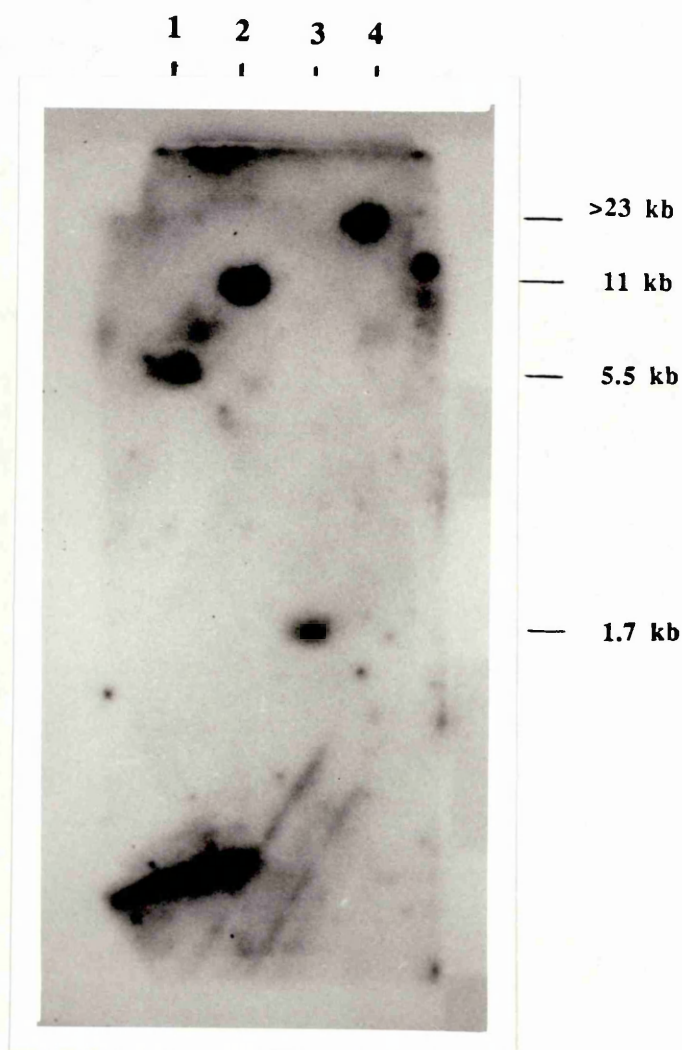


Figure 5.15 **Autoradiograph of a Southern blot of digested λ GW 4 DNA probed with the peptide oligonucleotide**

λ GW 4 DNA was digested with a range of restriction enzymes (Figure 5.11) and was transferred to a nylon filter by the method of Southern (section 5.3). The reaction with the peptide oligonucleotide was carried out under stringent hybridisation (60°C, 6 x SSC) and washing (65°C, 6 x SSC) conditions as described in section 5.6.3.

Lane 1 : *Sst* I
 Lane 2 : *Bam*H I
 Lane 3 : *Sal* I
 Lane 4 : uncut

The size of the single strongly hybridising band for each lane is indicated and listed (Table 5.2).

Table 5.2 **Size of hybridising restriction fragments obtained from digests of λ GW 4 DNA**

| Restriction enzyme(s) | Probe | Fragment size(kb) | Reference |
|-----------------------|--------------------|-------------------|-------------|
| <i>Sst</i> I | <i>N</i> -terminal | 5.5 | Figure 5.12 |
| " | peptide | " | Figure 5.13 |
| <i>Bam</i> H I | <i>N</i> -terminal | 11 | Figure 5.12 |
| " | peptide | " | Figure 5.13 |
| <i>Sal</i> I | <i>N</i> -terminal | 1.7 | Figure 5.12 |
| " | peptide | " | Figure 5.13 |

5.7 Discussion

Following digestion of *S.coelicolor* genomic DNA and hybridisation with various probes, restriction fragments were identified which could contain the *S.coelicolor* DAHP synthase gene. Following the failure to clone the gene from a genomic sub-library in vector pUC18, a *S.coelicolor* λ library was screened and two hybridising plaques isolated. One of these was characterised further by restriction analysis. The hybridising bands corresponds to those seen in the earlier genomic digest experiments.

The signal obtained from probing *S.coelicolor* genomic DNA is sufficiently strong at conditions of high stringency to conclude that this represents a genuine specific hybridisation event. The stringency of a hybridisation reaction is governed by the factors of temperature and salt concentration. The hybridisation temperature affects the stability of hybrid DNA. If it is too low then non-specific binding to non-target DNA may occur (Figure 5.2), if it is too high then the T_m , the melting temperature of the duplex, may be exceeded and hybrid formation prevented. Hybridisation protocols advise temperatures in the range 5-10°C below the T_m for a given duplex (Binnie, 1990). The salt concentration was kept high (6 x SSC ; 1 x SSC = 0.15 M-NaCl, 0.015 M-sodium citrate) to promote hybrid formation, then lowered (1 x SSC) during washing to remove probe bound non-specifically or weakly because of mismatches with the target DNA. These ionic conditions should favour the formation and detection of perfectly matched hybrids (Binnie, 1990).

Equations to calculate the T_m for any given duplex exist (Suggs *et al.*, 1981 ; Anderson and Young, 1985 ; Albretsen *et al.*, 1988). Factors influencing the T_m value include base composition (GC rich duplexes have a higher thermal stability than AT rich duplexes) and probe length. However, none of these equations are valid for the length and GC composition of the probes used in this study, or under the hybridisation conditions employed. In practice the T_m of an oligonucleotide-DNA duplex is determined empirically by carrying out a low stringency hybridisation and wash, and then making conditions

progressively more stringent (ie. temperature increased, ionic strength decreased) until a single specific signal is retained.

The more stringent the hybridisation and washing conditions employed the less one would expect mismatches between the oligonucleotide and the target DNA. Probes of similar length to those employed in this study have been used to isolate other genes from *S.coelicolor* (P.J. White, personal communication, 1990). In the case of the cloning of the gene encoding the enzyme 3-dehydroquinase, a 38-mer oligonucleotide containing two degeneracies was designed corresponding to *N*-terminal protein sequence. Stringent hybridisation and washing conditions, identical to those employed for the *S.coelicolor* DAHP synthase cloning, were used to isolate the gene. Subsequent sequencing of the DNA corresponding to the region complementary to the oligonucleotide probe revealed a single mismatch between the probe and target DNA sequences. By analogy, we expect there to be a similar absence of mismatches between the two homologous oligonucleotide probes employed in this study and the target DNA sequence corresponding to the DAHP synthase gene. Additionally, the fact that two probes have been used to isolate the same DNA fragment under conditions of such stringency make it highly unlikely that a DNA fragment has been isolated and cloned that does not correspond to the *S.coelicolor* DAHP synthase gene.

No strongly hybridising DNA fragments were identified following incubation of digests of *S.coelicolor* genomic DNA with a third oligonucleotide probe designed against a putative active-site sequence conserved between the *E.coli* and *S.cerevisiae* DAHP synthase isoenzymes (section 4.6.3). Following the failure of this work, the potato DAHP synthase sequence became available (Dyer *et al.*, 1990) and was shown to share only two amino acid sequence identities with the sequence used to design the heterologous oligonucleotide probe (Figures 4.8, 4.9), corresponding to residues 97 and 105 of the *E.coli* *aroG* sequence (Figure 5.16). In the five DAHP synthase sequences from *E.coli* and *S.cerevisiae* around 40% of the amino acid residues are common to all, but one can conclude from the potato sequence data that sequences from microbial and plant sources

Figure 5.16 **Alignment of DAHP synthase sequences from around the putative active-site residues from microbial and plant sources corresponding to the consensus peptide probe**

Sequences were aligned as described in section 2.22. Identical amino acids are indicated by bold type, a gap in the sequence by a dash.

| | | | |
|-----|---------------------------------|-----|--------------------------|
| 101 | | 115 | |
| | Q L P E Y P N Q E E - L R S V L | | <i>Solanum tuberosum</i> |
| 97 | | 112 | |
| | E K P R T T V G W K G L I N D P | | <i>E.coli aroF</i> |
| 95 | | 110 | |
| | E K P R T T V G W K G L I N D P | | <i>E.coli aroG</i> |
| 94 | | 109 | |
| | E K P R T T V G W K G L I S D P | | <i>E.coli aroH</i> |
| 105 | | 120 | |
| | E K P R T T V G W K G L I N D P | | <i>S.cerevisiae aro3</i> |
| 111 | | 126 | |
| | E K P R T T V G W K G L I N D P | | <i>S.cerevisiae aro4</i> |

exhibit a considerably lower level of sequence similarity than the microbial sequences.

Other microbial DAHP synthase sequences, including that of *S.coelicolor*, may exhibit a sequence similarity intermediate between the highly homologous *E.coli* and *S.cerevisiae* isoenzymes and the less homologous potato sequence. To ensure a reasonable chance of success when carrying out heterologous probing with relatively short oligonucleotides (eg. around 40 bp) where more than two nucleotide mismatches may be enough to prevent duplex formation at conditions of high stringency (R.D. Taylor, personal communication, 1991), one must be convinced that specific residues within a sequence are catalytically essential and that this sequence is conserved within a number of evolutionary distant species. With hindsight it can be seen that these criteria were not fulfilled for our heterologous probe.

We failed to isolate recombinants strongly hybridising to either of the homologous oligonucleotide probes from a genomic sub-library in vector pUC18. We had calculated, from the size of the *S.coelicolor* genome (8×10^6 bp) (D. Hopwood, personal communication, 1991) and an estimate of the enrichment factor achieved by agarose electrophoresis of digested DNA, that in screening 200 recombinants our sequence should be represented. We screened 570 recombinants in total without success. Agarose gel analysis of digested plasmid purified from a random selection of recombinants revealed that only 30% possessed an insert fragment of the correct size (data not shown). Others represented cloned partial digest fragments, with these inserts containing one or more internal *BamH* I restriction sites, despite our efforts to determine that digestion was complete. Additionally, control transformations with cut and dephosphorylated pUC18 which was ligated without insert and should not have produced any white recombinants did, at a frequency of 8%. Plasmid prepared from this type of white colony could no longer be cut by *BamH* I and we assumed that the free cohesive termini of the cut plasmid had been degraded and the recognition sequence lost. The cumulative effect of these two points reduces the number of representative 8-14 kb *BamH* I fragment containing recombinants

to around 170, concomitantly reducing the chances of having a reasonable chance of detecting the correct gene.

Two hybridising plaques were identified and purified from the *S.coelicolor* genomic library constructed in λ GEM-11. Given the size of the *S.coelicolor* genome (8×10^6 bp) and following the equation of Clark and Carbon (1976), one would expect to screen 5,756 independant recombinant plaques of the insert size 11-14 bp in order to have a reasonable chance (ie. a 99% probability) of including and identifying the desired sequence. From filters estimated to contain around 10,000 plaques we isolated two strongly hybridising plaques. This result would indicate that the amplification of the λ library had not resulted in serious distortion in the frequency of the occurrence of the target DNA sequence.

One of the hybridising plaques was characterised further. The hybridising restriction digest pattern of fragments obtained from λ GW 4 agrees with that previously obtained from experiments with genomic digests. The 11 kb *Bam*H I λ insert which hybridises to both homologous oligonucleotide probes is sufficiently large to encode the entire DAHP synthase gene. Further restriction digest mapping and sub-cloning is required before it can be concluded that the entire coding sequence lies within this insert.

Chapter 6 General discussion and future prospects

6.1 Introduction

The preceding chapters have described in detail the progress made in fulfilling the original project objectives. It has been shown for *S.coelicolor* :-

- (1) that only one DAHP synthase inhibitable by tryptophan is present.
- (2) that this enzyme possesses catalytic properties consistent with those reported from other sources.
- (3) that this activity is constitutively expressed.
- (4) that the subunit M_r determined corresponds to those found for other plant, fungal, streptomycete, and corynebacteria DAHP synthases.
- (5) that the limited amino acid sequence data obtained is dissimilar to the *E.coli* and *S.cerevisiae* isoenzymes.

Importantly, sufficient data has been obtained to enable us to conclude that in *S.coelicolor*, flux to aromatic amino acids, cofactors, and vitamins by way of the shikimate pathway is modulated by the end-product tryptophan acting via DAHP synthase.

6.2 Evolutionary implications of the regulation of DAHP synthase

DAHP synthase is a regulatory enzyme subject to possibly the most diverse pattern of feedback inhibition known for a single enzyme. This diversity of allosteric control was initially appreciated following a survey of species spanning 32 genera of microorganisms (Jensen *et al.*, 1967). This pattern, its use in delineating genealogical clusters, and its use in deducing evolutionary events, have already been described (sections 1.2.2.1; 1.2.2.1 (a)). It is pertinent to speculate as to how and why such a myriad of patterns arose.

The influence of the environment on the life-style of a microorganism and consequently on the control of DAHP synthase has already been discussed (section 3.13), with the conclusion that particular regulatory patterns may be rationalised as adaptations to specific niches. In many present day prokaryotes allosteric regulation is focussed at the initial pathway step and is not always developed at terminal-branch or mid-pathway steps. This form of pathway regulation appears adequate for many organisms (eg. cyanobacteria, corynebacteria, streptomycetes) (Jensen and Hall, 1982) who possess a single DAHP synthase.

If this method suffices for one group of organisms, why do other groups require two or more isoenzyme forms ? The enteric bacteria possess three isoenzymes of DAHP synthase, and it has been suggested that enteric enzymes may be inflexible with regard to altering substrate specificity. Hence, a mutation to produce a single activity cumulatively inhibited by two or more effectors would be less likely than a gene duplication event leading to an isoenzyme with altered effector sensitivity (Baumberg, 1981). Sequence evidence from the enteric isoenzymes certainly favours a common evolutionary origin (Pittard, 1987), with a single binding pocket modified to accomodate different effectors (Weaver and Herrmann, 1990). The other proposal of domain recruitment of effector binding regions now appears unlikely (Schultz *et al.*, 1984).

A further explanation for the apparently illogical plethora of regulatory mechanisms is that these present-day mechanisms may not reflect an optimal adaptation to current conditions, but are instead historical. A given feature may have been advantageous to an ancestor under very different circumstances. The system was retained with modifications superimposed, rather than the *de novo* development of a new optimal system (Baumberg, 1981).

Further speculation on evolutionary events leading to the current regulatory diversity should wait until many more DAHP synthase sequences from diverse organisms have been obtained.

6.3 Future work

The low levels of shikimate pathway enzymes found in most organisms has hindered the development of suitable purification procedures. Consequently, the application of chemical modification studies to identify catalytically important residues and elucidate possible reaction mechanisms has been delayed by a lack of protein. Where recombinant organisms, over-expressing individual shikimate pathway genes are available, rapid progress has been made in this field (eg. Kleanthous *et al.*, 1990). It should now be possible to overexpress the *S.coelicolor* DAHP synthase gene (using an appropriate streptomycete expression vector) in an amenable heterologous host (eg. *S.lividans*) and facilitate future physical and mechanistic studies.

A full analysis of the relationship between the *S.coelicolor* DAHP synthase and those from other organisms must await the determination of its full nucleotide sequence. From the data we have already obtained it is likely that the *S.coelicolor* DAHP synthase sequence may be dissimilar to the highly homologous *E.coli* and *S.cerevisiae* sequences. The clustering of antibiotic biosynthesis genes has been discussed (section 1.10.1). Additionally, other biosynthetic genes may also be clustered in streptomycetes. The clustering of three tryptophan biosynthesis genes on a 7.2 kb sized DNA restriction fragment in *S.griseus* IMRU 3570 (Lezcano *et al.*, 1990) has been reported, as has the localisation of three shikimate pathway genes from the corynebacterium, *Brevibacterium lactofermentum*, to an 8.5 kb DNA fragment (Matsui *et al.*, 1988). It would therefore be of interest to determine the nucleotide sequence up and downstream of the DAHP synthase coding region, in order to identify any other protein encoding regions and to determine by sequence comparison analysis the possible identity of these genes.

The study of metabolic flux in all organisms has been revolutionised by the application of flux control theory (Kacser and Burns, 1973), which provides a framework for the rational

manipulation of the flow of intermediates through a pathway. Each catalytic step in a pathway possesses a unique 'flux control coefficient' which can be regarded as a measure of the importance of that step in controlling overall flux through the pathway. This coefficient can be measured by varying a particular enzyme level and measuring its effect on pathway intermediates and end-product formation. In practice this is usually achieved by placing a cloned gene under the control of a modulatable promoter.

Although no single biosynthetic step is solely responsible for control of flux, a few enzyme catalysed steps may be expected to be most important. In the case of the shikimate pathway, DAHP synthase would be expected to be such an enzyme. Using the cloned *S.coelicolor* DAHP synthase gene, analysis of the effect of varied amounts of activity on flux through the shikimate pathway during growth and antibiotic formation could be determined. These studies could also be undertaken using *S.coelicolor* recombinants constructed to produce aromatic containing secondary metabolites. The ultimate objective in obtaining these data would be to remove any metabolic 'blocks' which could reduce maximal antibiotic production.

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